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Syverton Memorial Symposium

ANALYTIC CELL CULTURE

APRIL 1962

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PUBLIC HEALTH SERVICE
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Bethesda 14, Maryland

UNITED STATES OF AMERICA

Syverton Memorial Symposium

Analytic Cell Culture

Sponsored by
Tissue Culture Association
and
Cell Culture Collection Committee of the Viruses
and Cancer Board
National Cancer Institute
National Institutes of Health, Bethesda, Maryland
Held June 6 and 7, 1961
Detroit, Michigan

Symposium Chairman
Charity Waymouth, *Ph.D.*
President, Tissue Culture Association

Edited by
Robert E. Stevenson, *Ph.D.*

Acknowledgments

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The editor wishes to express his sincere appreciation to the staff of the Journal of the National Cancer Institute for their labor and interest in preparing this monograph. To Mrs. Sally Miles of the Viruses and Cancer Program, a special note of thanks is due for her excellent assistance above and beyond the call of duty.

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The Cell Culture Collection Committee felt that this symposium would be a fitting and tangible memorial to Dr. Syverton, reflecting not only his broad interests in cell biology and microbiology but also in attempting, as he did, to bridge some of the disciplinary areas which have become more parochial with increasing specialization.

Dr. Syverton was requested to organize and chair a symposium on Analytic Cell Culture as part of the 1961 Tissue Culture Association meeting, but at the time of his death in January, 1961, plans were incomplete. Dr. Charity Waymouth, Dr. Donald Merchant, Dr. William Scherer, and Dr. Robert Stevenson were asked to complete the program. Insofar as possible, Dr. Syverton's plans were carried out, additions were made and some changes occurred due to prior commitments of invited participants.

At the meeting, many tributes were paid to his memory and the bibliographies of many of the papers attest to his contributions in a diversity of subjects. His eagerness and enthusiasm were contagious and his quest for understanding the problems of cancer sincere.

"To travel hopefully is a better thing than to arrive, and the true success is to labor."

JEROME T. SYVERTON

Late Professor and Head
Department of Bacteriology
University of Minnesota
School of Medicine
Minneapolis, Minnesota

Born March 29, 1907; Died January 28, 1961; Father: John Syverton; Mother: Thea Nelson
Birthplace: Courtenay, North Dakota
Married Mildred Sloulin, June 26, 1932
Three children: Jane, Gail, and Laurie

A.B., University of North Dakota, 1927
B.S., University of North Dakota, 1928
M.D., Harvard University School of Medicine, 1931

Academic Appointments

University of North Dakota: Instructor in Bacteriology, 1928.
Duke University Hospital: Intern and Assistant Resident in Medicine, 1931–32.
Rockefeller Institute for Medical Research: Assistant in Pathology and Bacteriology, 1932–34.
Vanderbilt University School of Medicine: Visiting Associate Professor of Pathology and Bacteriology, April–October, 1942. (Sabbatical leave from the University of Rochester School of Medicine.)
University of Rochester School of Medicine and Dentistry: Instructor in Bacteriology, 1934–37; Assistant Professor, 1937–39; Associate Professor, 1939–47.

Louisiana State University School of Medicine, New Orleans: Professor of Microbiology and Head of the Department of Microbiology, 1947–48.
University of Minnesota School of Medicine, Professor and Head of the Department of Bacteriology, 1948–61.

Military Service

Medical Corps, United States Naval Reserve, 1941–47, active duty from April 27, 1944 to January 10, 1946, with an appointment as visiting investigator, hospital of the Rockefeller Institute, April–November, 1944; and foreign duty with Naval Medical Research Unit 2 in the Pacific Theater until return to inactive status.

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American Academy of Microbiology
Society of American Bacteriologists
American Society for Cell Biology
International Society for Cell Biology
American Society for Clinical Investigation
American Association for Cancer Research
Society for Experimental Biology and Medicine
American Association of Immunologists
American Epidemiological Society
American Association of Pathologists and Bacteriologists Executive Council, 1959–66
American Society for Experimental Pathology
American Society for Tropical Medicine
Central Society for Clinical Research
American Association for the Advancement of Science

MEMBERSHIP IN MEDICAL AND SCIENTIFIC SOCIETIES

- AMERICAN MEDICAL ASSOCIATION
- TISSUE CULTURE ASSOCIATION
- NEW YORK ACADEMY OF SCIENCES
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- COMMITTEE ON ENTEROVIRUSES, NATIONAL FOUNDATION AND THE NATIONAL INSTITUTES OF HEALTH, 1954–.
- ADENOVIRUS COMMITTEE OF THE NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, 1960–63.
- ADVISORY COMMITTEE ON RESEARCH ON THE ETIOLOGY OF CANCER, AMERICAN CANCER SOCIETY, INC., 1957–61 (CHAIRMAN, 1960–61).
- CELL CULTURE COLLECTION COMMITTEE, NATIONAL CANCER INSTITUTE (CHAIRMAN), 1960–64.
- VIRAL AND RICKETTSIAL REGISTRY, EXECUTIVE COUNCIL, 1959–62.

RECIPIENT OF THE LILLY AWARD FOR RESEARCH IN BACTERIOLOGY AND IMMUNOLOGY, 1938
RECIPIENT OF THE COMMONWEALTH FUND AWARD FOR CREATIVE WORK, 1957–58
DIPLOMATE OF THE NATIONAL BOARD OF MEDICAL EXAMINERS
LICENSED TO PRACTICE MEDICINE IN MINNESOTA AND NEW YORK STATE

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Session I

Preservation, Characterization, and Supply of Certified Cultures

Chairman: George O. Gey, *M.D., Johns Hopkins Hospital, Baltimore, Maryland*

Program on Characterization and Preservation of Animal Cell Strains[1]

WILLIAM F. SCHERER, ***Department of Microbiology, University of Minnesota, Minneapolis, Minnesota***

IN DECEMBER, 1959, on recommendation of the Viruses and Cancer Panel, the National Advisory Cancer Council of the National Cancer Institute appointed Dr. J. T. Syverton chairman of a committee to coordinate and supervise establishment of a national animal cell-culture collection and to encourage research on characterization and preservation of animal cell strains. In May, 1960, a grant was initiated to support committee activities. The Committee consists of investigators from actively cooperating laboratories and representatives from the Viruses and Cancer Panel, National Advisory Cancer Council, Study Sections (Cell Biology, Pathology, Virus, and Rickettsia), Naval Medical Institute, and National Cancer Institute. Special consultants also participate in meetings, and information concerning Committee activities is made available to other interested organizations. The Committee has met five times to date: March, June, and November, 1960, and January and June, 1961.

Current programs of the Committee are directed toward:

1. Establishing criteria for *characterization* of animal cell strains based on research by interested investigators.
2. Defining and recommending procedures for *propagation* and *preservation* of cell strains for use by cooperating laboratories and repositories.
3. Instituting procedures for *receipt, registration,* and *distribution* of cell strains by a repository.

To date, tentative culture-collection procedures have been conceived, including initial deposition of prototype cells at the American Type Culture Collection Repository. Candidate cell strains are requested, or considered, from originating laboratories or other laboratories recommended by originators. When possible, the original investigator is asked to aid in establishment of standards for his cells. Through the cooperating laboratories at the Child Research Center of Michigan, South Jersey Medical Research Foundation, and American Type Culture Collection, characterization, propagation, and preservation of cells are facilitated. A card system for recording data concerning cell strains has been developed. This is comprised of fourteen 4 × 6-inch cards which record information for each cell strain concerning its acquisition, shipping, history, bibliography, cultural procedures, viral susceptibility, preservation, bacteriologic analysis, decontamination history, constitutional characteristics, heterotransplantation data, and response to environment. The Committee has also investigated apparatus for incubation of cells, sealing of ampules, and storage and transportation of frozen cells. Initial criteria for cell characterization include freedom from microbial contamination, establishment of species of origin, chromosomal picture, viral susceptibility, and growth properties *in vitro* and *in vivo;* later other biologic, morphologic, immunologic, and biochemical characteristics of cells may be added.

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[1] Presented at the Syverton Memorial Symposium on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

The activities of the Committee from its inception until last January were spearheaded by Dr. Syverton. With his vigorous support, the Committee's program moved ahead rapidly. He was an outstanding scientist and teacher, as shown by the fact that over the past 12 years more than 65 students and postdoctoral fellows have studied with him. It was his exceptional ability to stimulate by leading rather than by pushing that made him so much in demand for activities such as the one of this Committee. Perhaps no better tribute can be made to Dr. Syverton than that expressed by Dr. Richard Shope, when he spoke to our medical students in February of this year: "Dr. Syverton was a fine scientist, a gentleman, and a grand person to have as a personal friend. I think that you were fortunate indeed to have had him as one of your professors for as long as you did."

DISCUSSION

Role of the American Type Culture Collection in Mammalian Cell Line Preservation: Cell Culture Collection Committee Meeting. WILLIAM ARTHUR CLARK, *Director, American Type Culture Collection, Washington, D.C.*

The American Type Culture Collection (ATCC) is a private, nonprofit corporation founded in 1925 and devoted to the collection, long-term preservation, and dissemination of authentic strains of microorganisms and other biological materials such as viruses and tissue-culture cell lines, and to research in preservation, characterization, and classification of such materials.

The Collection is governed by a Board of Trustees made up of representatives from the following societies concerned with microbiology:

American Society for Microbiology
American Phytopathological Society
American Association of Pathologists and Bacteriologists
American Association of Immunologists
Genetics Society of America
American Society of Zoologists
Mycological Society of America
National Research Council—National Academy of Sciences
American Institute of Biological Sciences

The activities of the ATCC are carried out under a Director and his curators, each of the latter in charge of a specific collection.

In 1959, the ATCC was asked by representatives of the National Cancer Institute to consider the establishment of a tissue culture cell line repository for long-term preservation and distribution of characterized cell lines. The ATCC accepted this challenge. It was reorganized and is now being rehabilitated to meet the demands of modern microbiology. An intensive drive was undertaken for funds to build suitable facilities for carrying out its program properly. The drive was successful, and funds were made available by the National Institutes of Health, National Science Foundation, and private industry to build the permanent facilities, to be completed by 1963.

Dr. John Shannon from the University of Nebraska will be the Curator of Cell Lines. In addition to the development of the cell line repository, research will be done by the ATCC on characterization and preservation of cell lines. Interested laboratories are invited to cooperate in this effort.

The ATCC now has limited facilities for long-term preservation of cell lines at liquid nitrogen temperatures. Cell lines considered for long-term preservation in the repository will be proposed either by the investigator or members of the Cell Culture Collection Committee for long-term preservation. If they are accepted by the Committee, such lines will be submitted to a cooperating laboratory for characterization, expansion, freezing, and deposit in the repository. Some 200 or more ampules of frozen material will be deposited as seed stock and kept at liquid nitrogen temperatures. Ampules of seed stock will be thawed, expanded, and refrozen in quantity for distribution, as needed.

As a service to the investigator, the ATCC is also working on plans for furnishing "safe deposit boxes" and distribution services for cell lines not in the repository.

Freezing of Living Cells: Biophysical Considerations [1]

H. T. MERYMAN, *M.D., Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland*

SINCE ALL biochemical reactions proceed at a temperature-dependent rate, the most important effect of reducing the temperature of living tissues is to reduce the rate of biochemical reactions. If this were the only effect of temperature reduction, the attainment of suspended animation at very low temperatures would be a simple matter. Unfortunately, at some temperature below 0° C water begins to crystallize out of solution, which creates a multitude of undesirable circumstances leading to injury and death of the cell (*1*). With the exception of microorganisms and certain plant cells that have become especially adapted to withstand low temperatures, living cells and tissues are generally killed by freezing. It should be recognized at the outset that, despite the complex biochemical and biophysical milieu, the freezing of water is a very straightforward physical phenomenon in which pure water separates from solution and is isolated throughout the tissue as inert foreign bodies.

When freezing is slow, the ice crystals invariably form outside the cells. As water is removed from the extracellular solution to form ice, this solution becomes concentrated and water will pass from within the cell to be added to the crystal. The ultimate effect is the removal from solution of all freezable water. Not all water in a biological solution is freezable. Water which is adsorbed to proteins, involved in the hydration of sugars and salts, and certainly that involved in hydrogen bonds, will not freeze. In blood the unfreezable water comprises about 8 percent of the total water.

When over 90 percent of the water in a tissue or cell suspension is removed from solution to form ice, the histological distortion is extreme and justifies the original theory of mechanical destruction from ice-crystal growth. However, this theory was inconsistent with reports from experimental frostbite research that tissue can withstand freezing if the exposure time is not excessive. This evidence, fully supported by subsequent studies, showed that the mechanical disruption caused by extracellular ice crystals is not necessarily lethal. The cells are apparently able to plasmolyze to an extreme degree without mechanical injury and, on thawing, to rehydrate and resume normal function.

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[1] Presented at the Syverton Memorial Symposium on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

To explain the injury that does occur from freezing, one must look beyond the mechanical effects of ice formation. The next best suspect is the extreme concentration that results when water is removed from the tissue solution. Over the past 10 years the theory of freezing injury due to biochemical denaturation from solute concentration has been quite serviceable. Although it is becoming increasingly obvious that this theory too needs modification, it is still an entirely workable and useful conceptual picture. To discuss the various means of preventing freezing injury we can adopt the simplified picture of ice crystals forming in the extracellular solution, withdrawing water both from within and without the cell, and of passive collapse of the cell between the ice crystals without mechanical injury but with denaturation proceeding with increasing concentration of cell solutes. This denaturation will proceed at a temperature-dependent rate, more rapid at higher temperatures and approaching a standstill at very low temperatures.

If the rate of freezing is accelerated, the ice crystals are smaller and more numerous. When the rate of cooling becomes exceedingly high, crystals will start to form inside as well as outside the cells. The formation of intracellular crystals appears uniformly lethal, probably due to mechanical destruction of the complex internal structure of the cell. Attempts to prevent mechanical injury by freezing so rapidly that ice crystals do not grow large enough to be significant have been unsuccessful, whether because of the difficulty in attaining adequate freezing and thawing rates or because of other factors is not known.

Based on these concepts of the mechanism of freezing injury, attempts to circumvent injury lead to paradox. If the injury to cells from slow freezing with extracellular ice crystals is biochemical in nature, one would expect to be able to freeze slowly but reach a much reduced temperature where biochemical activity is suspended before the development of irreversible injury. This cannot be done *in vitro* and slow freezing inevitably results in the loss of virtually all viability. As the rate of freezing is accelerated to reduce the opportunity for denaturation, the range of intracellular ice-crystal formation is reached and mechanical injury becomes dominant. The solution to this dilemma was found with the discovery of the protective powers of glycerin. This compound was shown to prevent freezing injury to bull sperm and erythrocytes when present in concentrations ranging from 10 to 20 percent. The explanation for the peculiar virtue of glycerin presumably lies in its ability to prevent water from freezing. Each molecule of glycerin can bind 3 molecules of water. This glycerin-water complex appears to function as a solvent for salts. The net result of the addition of glycerin to a tissue solution is a reduction in the amount of water frozen and, hence, a reduction in the final concentration of tissue solutes. This reduces the rate of denaturation and permits a tissue to be frozen slowly without rapid destruction. Since the discovery of glycerin, other materials have been found to have similar value as protective agents. Any satisfactory material for this purpose must fulfill three requirements: It must 1) bind water, 2) diffuse freely through cell membranes, and 3) be nontoxic. The use of glycerin as a protective agent has been extended to virtually every tissue of the body—enabling them to be frozen, held at temperatures sufficiently low to prevent change, and be subsequently thawed with good recovery.

Rapid freezing, contrary to popular impression, has not been used successfully in the freezing of any mammalian tissue with the single exception of erythrocytes which, either because of their small size or complete lack of internal structure, will survive sufficiently rapid freezing to enable survival in the absence of glycerin. Even red cells, however, are destroyed if the rate of freezing becomes excessively rapid.

The rationale of slow freezing with glycerin for the preservation of tissue-culture cells can now be analyzed on the basis of the foregoing discussion. The cultured cells must be equilibrated with glycerin which must penetrate the interior of the cell. The freezing rate must be slow to prevent the formation of intracellular crystals and also to permit the readjustment of water and solutes across the cell membrane as external ice is formed. Once all the freezable water has been removed, the temperature must be lowered sufficiently to prevent chemical activity. After thawing, the cells must be returned to a glycerin-free medium. The various steps in the procedure are discussed in sequence.

INTRODUCTION OF GLYCERIN

One of the specifications of a satisfactory protective agent is that it be freely permeable through the cell membrane. Glycerin appears in general to pass readily through cell membranes, but there is substantial evidence that its rate of transfer varies from tissue to tissue. Figures for the rate of transfer of glycerin through red-cell membranes (*2*) show that the penetration of glycerin through the membrane of the red cell is 50 times faster in man than in dog and 170 times faster in man than in sheep. Other animals with slow glycerin transfer into the red cell are cat, pig, and ox, while rat, mouse, rabbit, and guinea-pig red cells permit relatively rapid transfer. To protect the cells during freezing, the glycerin must be present both inside and outside the cell so that the time required for the entry of sufficient glycerin into the cell will depend upon the permeability of the membrane, the external concentration of glycerin, and the mass of the piece of tissue to be frozen. Very successful results have been obtained in some tissue cultures with as little as 5 percent glycerin. This suggests that the necessary concentration of intracellular glycerin need not be greater than this. The need for higher concentrations of glycerin for protecting other tissues may reflect poorer membrane permeability requiring a higher concentration gradient. There is also the advantage in the tissue culture that it consists of a single layer of cells as opposed to a chunk of tissue in which diffusion to the interior may be slow and therefore require a higher concentration gradient. Probably the easiest way to gain a rough index of membrane permeability to glycerin is by direct microscopic observation of the cells as glycerin is added to the medium. As long as the external and internal glycerin concentrations differ, there should be a corresponding difference in cell size so that the speed with which equilibrium is established can be estimated visually. Failure to achieve equilibrium, or at least to reach a satisfactory internal concentration of glycerin, can be a cause of failure in achieving good viability after freezing and thawing. Recent results with dimethylsulfoxide indicate that it may be superior to glycerin because of its more rapid passage through cell membranes. Dimethylsulfoxide is used in about the same percentage concentrations as is glycerin.

FREEZING

An excessive rate of temperature reduction at temperatures above freezing has occasionally been reported as a cause of cell injury. In general, however, these episodes appear to have involved rather special circumstances, and as yet there is no evidence that this is a general danger. As freezing begins, ice crystals form in the extracellular solution and the extracellular solute concentration gradually increases. This will result in an increase in the osmotic pressure of the extracellular solution as well as an increase in the glycerin concentration. One cannot state *a priori* exactly what transfers will take place across the cell membrane. Obviously water will pass out, and there may be a movement of glycerin and other solutes into the cell. In general, one would presume that the cell would shrink in size during the freezing process if a lag existed in equilibration of its osmotic pressure with the exterior. Certainly the final result when freezing is complete is a very extensive plasmolysis. Regardless of its specific nature, there will be a substantial amount of transfer across the membrane during freezing, a transfer taking place at a temperature somewhere below 0° C where the viscosity and resistance to diffusion is greatly increased. The reduction in diffusion rates necessary for the maintenance of equilibrium is a probable explanation of the importance of good control over the rate of temperature fall during freezing. A temperature fall of 1° C per minute was found to be the optimum rate for the freezing of bull sperm and red blood cells. The experience accumulated so far in glycerin freezing of a variety of other tissues indicates that, although in some cases other rates may produce equally good results, sometimes the results are not as good and in no event have other rates produced better results. This is very empirical evidence, but until better control studies are undertaken there appears to be no reason to depart from the 1° per minute cooling rate. It is frequently pointed out, in criticism, that the actual temperature of the specimen immersed in a bath falling in temperature at the rate of 1° per minute does not follow the bath temperature exactly, but shows a temperature plateau as freezing occurs, followed by an accelerated fall to catch up with bath temperature. This is a perfectly valid observation, but since the effectiveness of this system is an empirical observation, the criticism is not meaningful. Experience so far indicates simply that freezing in a bath falling in temperature at 1° C per minute has the best chance of yielding good results regardless of the actual temperature sequence in the specimen itself.

STORAGE

Any changes in the specimen during storage will take place at a temperature-dependent rate. One can therefore say with absolute confidence that storage changes can be prevented by maintenance at a sufficiently low temperature. One frequently hears it said that a certain tissue does not "freeze well." Further investigation may reveal that though the material survived satisfactorily after a few weeks of storage, there was a significant loss of viability after several months. One must carefully distinguish between injury resulting from the freezing and thawing sequence and injury occurring during storage. Freezing and thawing injury may indeed be a function of the particular tissue line and the technique used, but storage injury can result from only one thing: inadequate storage temperature. In practice good long-term preservation can only be guaranteed when the storage temperature is below −100° C. Dry ice at −79° C appears adequate for fairly long-term storage of certain materials, but as a standard storage temperature for all applications it is unsatisfactory. Very low temperatures can be attained by commercial mechanical refrigeration equipment, but one should recognize that unusual precautions must be taken against electric or mechanical failure. When the stored material has great value, particularly when its value increases with storage time, liquid-nitrogen storage is recommended as the only completely safe method. Liquid nitrogen has a temperature of nearly −200° C, thus giving a very substantial margin of safety in terms of storage temperature. It is stored in insulated containers at atmospheric pressure. Containers designed specifically for the storage of biological material are commercially available. The larger containers will last for weeks or months on one filling of nitrogen so that a regular filling schedule virtually eliminates the danger of a lapse in refrigeration.

THAWING

The thawing of frozen material has been curiously neglected as an area for study. Both the theories of mechanical destruction from ice and chemical destruction due to solute concentration place the entire blame for injury on the freezing portion of the process. Again research on frostbite is forcing a reappraisal of current theory. Past experience has indicated that seriously frozen extremities allowed to thaw spontaneously in a warm room, wrapped in blankets, or, even worse, rubbed in snow, inevitably develop extensive tissue loss. Recent studies (*3*) have shown that when the frozen extremities are thawed by immersion in 42° C water, tissue loss can be avoided despite long exposure to freezing. This is a freezing which, on the basis of theory, should be exceedingly injurious. It is a very slow freezing with storage at a relatively high temperature and would be completely lethal if performed *in vitro.* Yet an alteration in the thawing procedure has enabled the survival of the tissue. This is not to say that the simple device of rapid thawing will enable tissues *in vitro* to survive freezing in the absence of glycerin. Obviously the conditions of freezing are different and, in particular, the postthawing circumstances of the environment *in vivo* cannot be ignored.

Recent studies on the preservation of whole human blood by rapid freezing in the absence of glycerol have also demonstrated the importance of the thawing process (*4*). Cells frozen at a constant rate and thawed in a 20° C bath show nearly 100 percent hemolysis, but when the thawing solution is increased in temperature to 45° C, the hemolysis is reduced to around 30 percent. When the thawing solution is 45° C and also contains a 20 percent solution of the plasma expander, polyvinylpyrrolidone, hemolysis is reduced to about 5 percent. It is thus inescapable that action taken during the thawing can have a significant effect on the results of freezing and thawing, and we cannot assume that the injury from freezing is irreversible or that the present theory of freezing injury is irrefutable. The thawing of tissue-culture suspensions frozen in glycerin should be as rapid as possible. If it is practical to do so, rapid thawing can best be achieved by the introduction of warm medium containing glycerin directly into the ampule containing the frozen culture.

HANDLING AFTER THAWING

After the cells have been thawed, they are still suspended in glycerin and subsequent use will of necessity be in glycerin-free media. The same precautionary remarks regarding the glycerolization and freezing apply in reverse at this juncture. If cells containing internal glycerin are suddenly exposed to a glycerin-free medium, the resultant osmotic shock will depend on the degree to which glycerin can pass the membrane. Glycerin is often referred to as having no osmotic properties, but this statement must be qualified by a time factor. Glycerin has no osmotic properties as long as it diffuses through the membrane rapidly enough to maintain an equilibrium between exterior and interior. If the change in concentrations is sufficiently sudden or if the transfer of glycerin across the membrane is slow, very real osmotic effects will result. During reconstitution the glycerin is inside the cell, and changes in concentration will produce not shrinkage but swelling from which rupture may result. It is probable that a substantial amount of cell loss after freezing and thawing in glycerin occurs through a failure to recognize the osmotic hazards of sudden resuspension in glycerin-free media. Where serious losses are experienced, it would be wise to consider the possibility of this sort of injury and to see whether improved results might be obtained by a gradual or stepwise reduction in the glycerol concentration.

* * *

It should be evident from this summary that the technique of tissue-culture freezing in glycerin as now practiced is largely empirical and that there will inevitably be great improvement in the results of the future. It does not seem unlikely that the need for glycerin can eventually be obviated, and the circumstances of the Eskimo's toe that survived 2 days at —30° without tissue loss can be duplicated for the preservation of isolated mammalian cells and tissues.

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DISCUSSION

Mechanisms of Injury at Subzero Temperatures.

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Two consequences of lowered temperatures are potentially injurious to cells and tissues. The first is a decreased rate of all chemical and most physical reactions. Since the rates of the various reactions involved in cellular processes are not likely to decrease to the same extent, the resulting biochemical unbalances could conceivably be fatal. Although logical, this hypothesis, in general, is not supported by experiment. Most cells are harmed not by lowered temperatures *per se*, but by ice formation, which is the second consequence of lowered temperature. With yeast cells, for example, none are killed by exposure to —15° C when the surrounding water is unfrozen, but some 75 percent are killed when the surrounding water is frozen (*1*).

To say merely that death is associated with ice formation helps little in deciding on the precise physical-chemical mechanisms involved, and helps little in deciding what countermeasures are to be taken to achieve successful low-temperature preservation of cells and tissues. The consequences of ice formation are of two sorts, either one of which is potentially lethal. First of all, there are the direct effects of the solidification of water into crystals possessing properties quite different from those of the liquid. Secondly, there are the indirect effects arising from the fact that freezing removes water from a solution and, thus, produces an increase in the solute concentration of the remaining unfrozen solution.

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[1] Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

Dr. Meryman has already discussed both of these and indicated the expected sorts of cellular damage produced by the direct and indirect effects. What I would like to emphasize is that the specific steps required to prevent low-temperature death of a particular cell type may depend critically on just which factor is primarily responsible for death. Let me illustrate. If injury is caused by the indirect effects of high concentrations of electrolytes, one can make the following predictions:

1) Slow cooling should be harmful. The detrimental effects of concentrated solutes would be primarily chemical and, therefore, should increase with increasing length of exposure to the higher subzero temperatures. Slow cooling, of course, produces longer exposure than a higher cooling rate.

2) The injury produced by storage at a given temperature should increase progressively with increasing length of storage, the reason being that just stated under 1).

3) The addition of electrolytes to the suspending medium would probably increase injury and, at any rate, would not be expected to protect cells from injury.

4) The removal of water by means other than freezing should be at least as harmful as its removal by freezing. Room temperature dehydration, for example, would not only produce a concentration of electrolytes, but would expose the cells to these higher concentrations at relatively high temperatures.

5) To protect the cells from injury, an additive would have to be capable of penetrating the cells. A low molecular weight, nonpenetrating compound would tend to dehydrate cells by osmotic withdrawal of water and such dehydration, as already indicated, should be injurious. On the other hand, if the additive remained extracellular and were of sufficiently high molecular weight to have negligible osmotic effect, it is difficult to see how it could prevent the intracellular solutes from concentrating during freezing and exerting a detrimental effect.

With cells of the yeast *Saccharomyces cerevisiae,* none of these predictions hold true. In the first place, slow cooling is considerably less harmful than rapid cooling (50% vs. 0.01% survival). Secondly, injury does not increase with length of storage at −30° C (all death occurs in the 1st minute of exposure and none in the ensuing 90 minutes). Thirdly, the presence of electrolytes such as NaCl and $CaCl_2$ in the suspending medium not only is noninjurious, but actually reduces low-temperature injury (*1*). Fourthly, dehydration of yeast from the liquid state at above-zero temperatures is not as harmful as exposing yeast to below-zero temperatures (*2*). Finally, freezing injury is reduced by the presence of glycerol in the suspending medium (*3*) even when the length of exposure to glycerol prior to freezing is too brief to permit significant permeation of this poorly penetrating solute (*4*). Therefore, death of these cells evidently is not the result of exposure to a high concentration of solutes; rather, it appears to be a consequence of some direct effects of ice crystals, and in particular of intracellular ice crystals. Such a hypothesis is compatible with the findings just enumerated, and based on these findings, one can see that the procedures which yield high survival are entirely different from those that would be suggested by the alternative hypothesis of death from exposure to concentrated solutions.

Even though some other species of fungi and bacteria respond similarly (*5*), the hypothesis of death from intracellular freezing does not appear applicable to all microorganisms. Cells of *Escherichia coli,* for example, apparently succumb to exposure to the high solute concentrations induced by freezing (*6*). Dr. Meryman pictures animal cells as facing a Hobson's choice: Rapid cooling initiates lethal intracellular freezing; slow cooling leads to death by denaturation from exposure to high salt concentration. From the pragmatic point of view, glycerol is a fortuitously suitable protective additive, for it can protect cells from either mechanism of damage. By virtue of its colligative properties, it reduces the concentration of other toxic solutes at given subzero temperatures, and by virtue of being a strong hydrogen bonder, it can reduce the total amount of freezable water and prevent freezing. But from the mechanistic point of view, the very omnipotency of glycerol makes it difficult to use the fact of glycerol protection in interpreting just what lethal mechanism glycerol is protecting against. This difficulty with respect to mechanisms may become pragmatically important in instances when glycerol is undesirable as an additive, *e.g.*, in freeze-drying or in human blood and bone marrow preservation. The search for other additives may then have to proceed on an empirical basis. With the last two mentioned tissues, the search has uncovered protective substances which would not be expected to be protective on the basis of the "concentrated solution" theory. These include inorganic and organic electrolytes, such as sodium nitrate and L-glycine which protect mouse bone marrow cells (*7*, *8*), and the large molecular weight compound, polyvinylpyrrolidone, which protects human red cells from hemolysis (*9*).

Such findings indicate, as Dr. Meryman pointed out, that the theory of injury from solute concentration is, in all likelihood, oversimplified, even when restricted to animal cells. It may be oversimplified in being an incomplete explanation of injury in animal cells in general; that is, death could be the result of a combination of mechanisms, only one of which may be exposure to concentrated solutes. Or it may be oversimplified in applying only to certain animal cells and not to others.

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Quantitative and Qualitative Preservation of Cell-Strain Characteristics [1,2]

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COLLECTION OF characterized cell strains must be based on the premise that cultured cells can be readily preserved and that the identifiable properties of such cells are not altered during preservation. Based on previous studies of biological applications of freezing and drying, Scherer and Hoogasian (*1*) preserved cultured mammalian cells by freezing. Subsequently, Stulberg *et al.* (*2*, *3*), Swim *et al.* (*4*), and Hauschka *et al.* (*5*) reported refinements or applications of the method. The successful efforts of these investigators depended on a common general procedure, *i.e.*, slow freezing of cells suspended in glycerol-serum media, storage at temperatures below −70° C, and rapid thawing. Thus within a fairly wide range of freezing rates, glycerol concentrations, and storage temperatures viable cells usually could be recovered. It soon became apparent, however, that experiences varied in a number of laboratories. These variations depended partly on the variety of freezing and storage procedures employed, the differences between cell strains in their ability to withstand freezing, and the criteria used to judge the efficiency of recovery of cells from the frozen state. Obviously, unless attention was given to such factors, quantitative recovery of viable cells would be unpredictable, and from a qualitative point of view, freezing *per se* might lead to selection of only certain types of cells or certain characteristics from a given cell population.

Accordingly, this paper describes our methodology for preparing and preserving reference seed stocks of cultured cell strains for characterization and certification. Data are presented on quantitative determinations for cell viability, which provide a means for the establishment of optimal conditions and control of preservation. It is shown that various strains possess individual viability and freezing characteristics, and that such differences between cell strains provide additional means for cell characterization.

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[3] The authors acknowledge the technical assistance of Frank Nestico

METHODOLOGY

To prepare and preserve cell strains for characterization and certification by the Cell Culture Collection Committee, it was necessary to develop procedures for obtaining suitable reference seed stocks, since samples of these stocks would be used by laboratories participating in the characterization program and the remainder would be placed in the permanent repository. Strains were received in central reference laboratories where they were subjected to the minimal amount of subculturing needed for preliminary determinations, such as verification of freedom from microbial contamination, verification of species of origin, essential growth characteristics, and morphologic studies. The next step involved the preparation and preservation of the final reference seed stock. The following methodology is concerned principally with the latter objective, and particularly with quantitative preservation of viability.

Culture procedures.—To carry out the present evaluations on a comparative basis, all strains were cultivated as monolayers with Eagle's minimal essential medium (MEM)[4] (*6*) containing 2.0 g per liter $NaHCO_3$, and were supplemented with "nonessential" amino acids in concentrations recommended by Eagle (*6*) and with 10 percent inactivated human serum (for human-derived strains) or 5 percent inactivated calf serum. Antibiotics were not used. Prescription bottles (Duraglas, Owens-Illinois) of various sizes were used for the monolayer cultures; 32-ounce prescription bottles were employed when cell harvests of a substantial size were desired. All cultures were incubated in a 5 percent CO_2-95 percent air atmosphere in the humidified constant-flow CO_2 incubator shown in text-figure 1. Cells were removed from the culture bottles by treatment of the unwashed monolayers with 0.25 percent trypsin in calcium-free Hanks' balanced salt solution at room temperature until cells were freed from the glass (3 to 10 min, depending on the cell strain).

Viable cell determination.—In this report, the term "viable cells" will arbitrarily be equated with cells that were unstained with trypan blue. We made all counts by mixing 1 ml of cell suspension with 0.5 ml of a 0.5 percent trypan blue solution and enumerating the stained and unstained cells in a hemocytometer. The mean of 4 replicate counts was used to determine the number of viable and nonviable cells per sample.

Plating efficiencies.—A modification of Puck's procedure (*7*) was employed. Cells harvested from monolayer cultures as just described were suspended and then appropriately diluted in complete growth medium. One ml of a selected dilution was added to 5 ml of medium (prewarmed at 37° C in an atmosphere of 5 percent CO_2) in a 60 mm tissue-culture plate.[5] Plates were incubated, undisturbed, in the CO_2 incubator for intervals separately established for each cell strain to obtain reproducible

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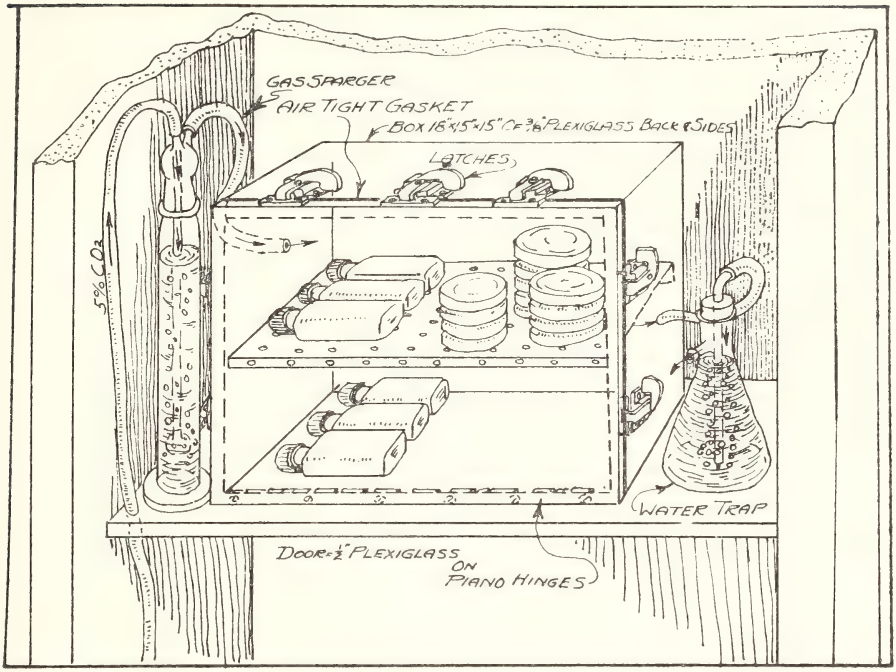
[4] The amino acids, vitamins, and salts were obtained as concentrates from Microbiological Associates, Inc., Bethesda, Md. The complete MEM was formulated in 10 liter amounts, sterilized by a closed system of filtration through 142 mm HA Millipore filters (Millipore Corp., Bedford, Mass.) into a 12 liter storage flask with a volumetric dispensing tube (Bellco Glass Co., Vineland, N.J.), and stored at 4° C.

[5] Tissue-culture dish, TCPD 6015, Falcon Plastics, Los Angeles, Calif.


GAS SPARGER
AIR TIGHT GASKET
BOX 18"×15"×15" OF 3/8" PLEXIGLASS BACK & SIDES
LATCHES
5% CO2
WATER TRAP
DOOR=½" PLEXIGLASS ON PIANO HINGES

TEXT-FIGURE 1.—Constant-flow CO_2 incubator for culturing cells in a humidified 5 percent CO_2-95 percent air atmosphere. Incubator designed for low consumption of CO_2 and for maximum humidity without condensation.

colony counts. These incubation periods ranged from 8 to 15 days depending on the cell strain. Ten plates per dilution were prepared and tested; the dilution selected for colony counts was one which produced 20 to 30 colonies per plate. Plating efficiencies were expressed as the ratio of number of colonies times the dilution : number of unstained cells per ml, and indicated as percent. At the end of the appropriate incubation period the medium was removed, and the colonies were examined and counted by visualization at low magnification.

Freeze-preservation procedure.—Although our preservation procedures were, in general, similar to those reported earlier (*3*), several technical modifications and improvements were introduced to handle cell strains for the cell-culture collection. The essential steps of the preservation procedure are:

1. Dispersion of cells: trypsinized monolayers.
2. Freeze medium: MEM, 80 percent; serum, 15 percent; glycerol, 5 percent.
3. Slow freeze: 1.8° C per minute past crystallization phase.
4. Storage temperature: $-165°$ to $-195°$ C (liquid nitrogen).
5. Rapid thaw: approximately 3° C per second.

Preparation of cells for freezing.—The various strains were cultured in a sufficient number of 32-ounce prescription bottles to provide approximately 5 to 10 $\times$ 10^8 cells for preparation of a single frozen seed stock. The medium was completely renewed 18 to 24 hours before being harvested. The cells were removed with trypsin solution, 20 ml per bottle culture, and the dispersed cells were centrifuged under refrigeration at 4° C in 125 ml conical centrifuge flasks (Bellco Glass Co., Vineland, N.J.) at 2000 rpm for ½ hour, and then pooled and resuspended in the "freeze medium." The latter consisted of Eagle's basal medium containing 0.3g $NaHCO_3$ per liter, 80 percent; serum, 15 percent; and glycerol, 5 percent, by volume. After a preliminary cell count, 3 to 5 $\times$ 10^6 viable cells were dispensed into 1 ml ampules. At this point, prefreeze viable cell counts and plating efficiencies were carried out.

Slow-freeze procedure and storage equipment.—The decision of the Cell Culture Collection Committee to use liquid-nitrogen refrigeration for permanent storage of certified cell strains necessitated the development of a standardized system suitable for the freezing and storage of at least 200 1 ml ampules of each strain included in the collection. The methods had to be readily adaptable to the preservation of smaller batches for various preliminary studies of each strain without alteration of the conditions of the freeze-preservation cycle. For these purposes an automatic controlled-rate slow-freeze unit [6] and a permanent storage unit,[6] both utilizing liquid nitrogen as the refrigerant, as well as accessory equipment for handling the ampules, were employed.

Because of thermal stresses involved in liquid-nitrogen refrigeration, precautions were taken with the type of ampule used and the manner of sealing. Thick-walled 1 ml ampules,[7] ordinarily used for preservation of bovine semen, were satisfactory for these purposes. These were prelabeled and sterilized (with no prior washing) in racks designed to hold 14 ampules for subsequent rapid filling. Care was taken to keep the ampule necks dry inside and out while being filled with cell suspensions. The filled ampules were tip-sealed in a semiautomatic apparatus,[8] each carefully examined for evidence of improper sealing, and mounted on stainless-steel canes,[9] each cane holding 6 ampules. The canes were then inserted into canister racks designed to fit the liquid-nitrogen slow-freeze unit.[6] This device was programmed to control the freezing of cells at predetermined optimal rates. The components of this system are illustrated in text-figure 2. For each freezing operation, 6 canisters[6] containing a total of 504 ampules (combinations of ampules with cell suspensions and media-filled "dummies") were placed in an empty 25 liter liquid-nitrogen refrigerator.[6] The attached freezer then vaporized liquid nitrogen, contained in a second 25 liter refrigerator, at a controlled rate, and transferred the cold vapor to the chamber containing the ampules. When the desired terminal temperature was reached through slow freezing, the ampule chamber was either automatically filled with liquid nitrogen or the ampules were transferred to the larger 300 liter liquid-nitrogen

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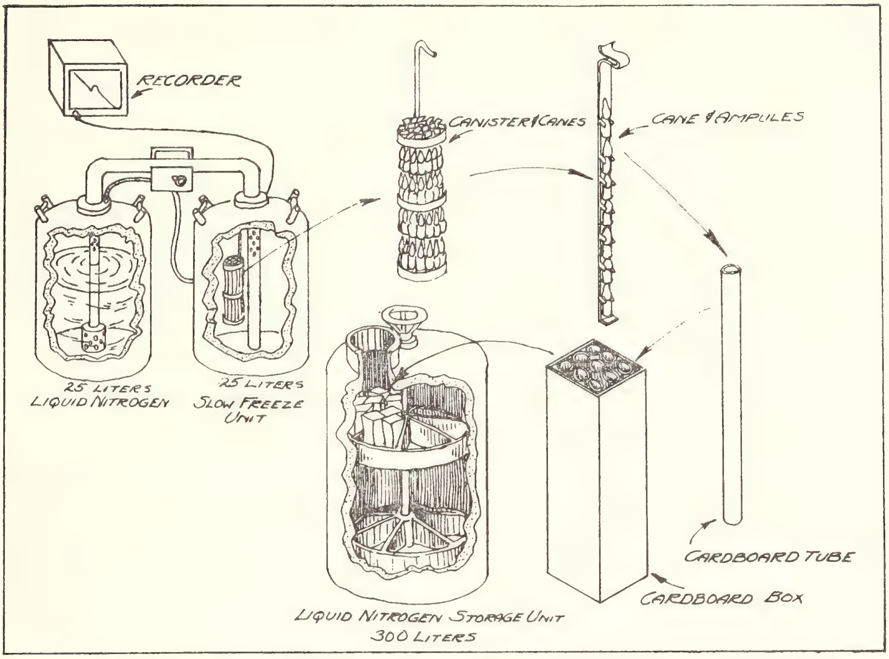
[6] The BF-1 bovine semen freezer, LNR 25 liter and 300 liter liquid-nitrogen refrigerators and canisters were obtained from the Linde Co., Cryogenic Division, 270 Park Ave., New York 17, N.Y.

[7] Insemination ampules, #6101-833, obtained from Wheaton Glass Co., Milleville, N.J.

[8] Labseal, HS-1, obtained from Popper and Sons, Inc., New York 10, N.Y.

[9] Canes with clips, cardboard protector tubes, and perforated cardboard containers were obtained from Frozen Semen Products, Inc., Breinigsville, Penna.


RECORDER
CANISTER & CANES
CANE & AMPULES
25 LITERS LIQUID NITROGEN
25 LITERS SLOW FREEZE UNIT
CARDBOARD TUBE
CARDBOARD BOX
LIQUID NITROGEN STORAGE UNIT 300 LITERS

TEXT-FIGURE 2.—Schematic arrangement of freeze-preservation apparatus.

storage apparatus.[6] For the latter purpose the canister racks were quickly placed in the upper compartment of the 300 liter refrigerator, where the canes were separately removed and inserted into precooled cardboard containers.[9] The containers were stacked on rotating trays, either immersed in liquid nitrogen or above the liquid-nitrogen level. In this arrangement each cane was readily accessible, since the top flange of the canes was labeled for quick identification, and individual canes could be removed from the refrigerator by lifting them directly out of the containers without disturbing the others. The prevailing temperature gradients, when the liquid-nitrogen levels were maintained between 75 and 150 liters, were between −165 and −195° C.[10] To maintain these levels 50 to 75 liters of liquid nitrogen were required once a week.

Freeze rates.—With each batch of cells frozen, a copper-constantan thermocouple, inserted into a pilot 1 ml vial, with leads to a recorder,[11] gave an exact record of the rate of temperature drop. A representative freeze rate cycle is shown in text-figure 3. Between 20° C and −10° C, the cells were cooled at a uniform rate of 1.8° C per minute. When the temperature reached −10° C, the contents of the ampules began to solidify and there was a short interval of temperature rise to −2.5° C

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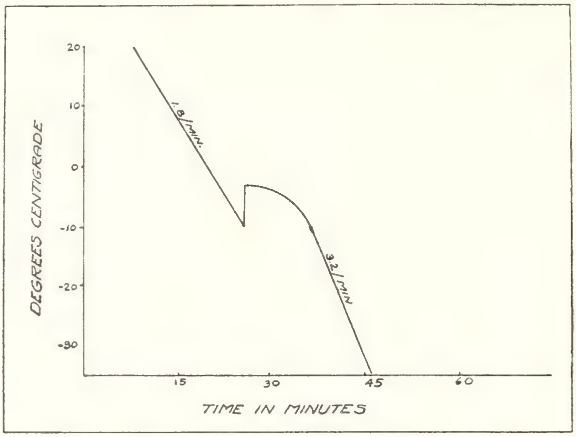
[6] The BF-1 bovine semen freezer, LNR 25 liter and 300 liter liquid-nitrogen refrigerators and canisters were obtained from the Linde Co., Cryogenic Division, 270 Park Ave., New York 17, N.Y.

[9] Canes with clips, cardboard protector tubes, and perforated cardboard containers were obtained from Frozen Semen Products, Inc., Breinigsville, Penna.

[10] Data supplied by Cryogenic Products Development Laboratory, Linde Co., Speedway, Ind.

[11] Minneapolis-Honeywell-Brown recorder that records on 10 inches of the chart width for 100° C temperature spread (−50 to +50° C). It is possible to interpolate changes of 0.5° C or better.



TEXT-FIGURE 3.—A record of the rate of temperature drop obtained with cells slow-frozen in the liquid-nitrogen controlled-rate apparatus.

due to the release of "heat of fusion." Once the temperature had returned to −10° C (in this system, after an interval of 12 min), the rate of cooling was 3.2° C per minute for the remainder of the freezing period.

Thawing and viability determinations on thawed cells.—Ampules taken from the liquid-nitrogen storage unit were agitated in a water bath at 37° C. Under such conditions, the contents of an ampule were thawed within 55 seconds. Viable cell counts and plating experiments were performed directly on thawed cells removed from the ampules.

EFFECT OF FREEZING AND THAWING ON VIABILITY

Our experiments provide examples of the effects of the described preservation procedures on the viability of various types of cells as measured by two quantitative criteria: exclusion of trypan blue and ability of individual cells to attach and multiply on glass or wettable plastic surfaces.

The cell strains are listed in table 1, together with data pertinent to their source. These strains were selected because they had one or more identifiable "marker" characteristics, *i.e.*, morphology, species-related antigens, chromosome constitution, tumorigenicity, or virus susceptibility spectrum. Verification of species of origin was accomplished by two procedures: the hemagglutination test of Brand and Syverton (*8*) and the direct fluorescent-antibody method reported by Stulberg *et al.* (*9*). All strains were examined for infection with bacteria, L-forms, or pleuropneumonia-like organisms (PPLO), and if present, such organisms were eliminated effectively with kanamycin (*10, 11*).

Table 2 records the results of an experiment to determine the effect of freeze preservation on strain HeLa 229. This strain, selected by the Cell Culture Collection Committee because it had been subjected to only a

Table 1.—Cell strains* studied for preservation characteristics

Cell strain	Species and tissue of origin	Characterizing properties	Investigators and references
HeLa 229	Human, cancer of cervix	Epithelial-like, heteroploid, species-related antigens, virus susceptibility	Gey, (#229, Scherer) (*12*)
Detroit-6	Human, sternal marrow	Epithelial-like, heteroploid, species-related antigens, virus susceptibility	Berman *et al.* (*13*)
Detroit-504	Human, teratoma of testes	Fibroblast-like, diploid, species-related antigens, virus susceptibility	Stulberg *et al.* (*2*)
S-180 II	Mouse, transplantable Sarcoma 180	"Fibroblast-like," tumorigenic, species-related antigens	Foley *et al.* (*14*)
LLC-MK_2 (NCTC 3196)†	Monkey, kidney	"Epithelial-like," nutritional requirements, species-related antigens, virus susceptibility	Hull (*16*), (NCTC 3196, Evans *et al.*) (*15*)

*Strains other than those originating in this laboratory were kindly supplied by the following investigators: HeLa 229, Dr. J. T. Syverton; S-180, Dr. G. E. Foley, Children's Cancer Research Foundation, Boston, Mass.; LLC-MK_2 (NCTC 3196), Dr. R. N. Hull, Eli Lilly Research Laboratories, Indianapolis, Ind.

†Sublines of this strain were examined both after propagation in synthetic and in serum-containing media.

TABLE 2.—Recovery of HeLa 229 cells from low-temperature storage as measured by trypan blue exclusion and plating efficiencies

Preparation	Weeks stored	Total cells/ml × 10^6	Viable cells (% unstained)	Viable cells (% recovered)	Plating efficiency (%)
Prefreeze		3. 6	98. 3		36. 4
Frozen-thawed	1	3. 6	93. 8	95. 4	N.D.
Frozen-thawed	2	4. 4	92. 4	94. 0	37. 8
Frozen-thawed	11	3. 5	95. 0	97. 6	39. 1

few subcultures since its preservation in 1954, was reactivated for the preparation of a seed stock for cell-culture collection. A single batch of 150 ampules (1 ml), each containing approximately 3.5 × 10^6 cells, was prepared according to the described protocols. The data in table 2 pertain to trypan blue staining and plating-efficiency determinations on this seed suspension just before it was frozen, and after it was removed from storage in the liquid-nitrogen refrigerator, for intervals up to 3 months, and thawed. The prefreeze determinations indicate that 98.3 percent of the total number of cells were unstained, and thus were presumed to be viable. Of these, 36.4 percent could attach to the plating surface and multiply, as exhibited by formation of macroscopic colonies under the described conditions. These values were consistent with viability counts and plating efficiencies previously observed for this strain during serial subculture that preceded the preparation of the cells to be frozen. Viability counts of cells taken directly from thawed ampules, after the specified periods of preservation, revealed that the survival of cells was uniformly high, with recoveries about 95 percent of those frozen. Of the viable cells recovered, their capacity for growth remained unaltered, in that the plating efficiencies were essentially the same as before freezing. Thus, under the conditions and criteria employed, the assessment of viability and growth potential of a cell strain before and after freezing may be readily accomplished.

It was of interest, therefore, to examine the variety of cell strains listed in table 1 in the same manner. Table 3 compares the viability and plating efficiencies of each of these strains before and after freezing. It can be seen that the prefreeze viability cell count of the Detroit-6 strain

TABLE 3.—Comparative viability data of several cell strains stored at low temperatures

Cell strains	Prefreeze viability determinations		Average recovery of cells from storage	
	Viable cells (% unstained)	Plating efficiency (%)	Viable cells (%)	Plating efficiency (%)
HeLa 229	98. 3	36. 4	95. 7	37. 8
Detroit-6	96. 9	53. 8	96. 7	55. 0
Detroit-504	95. 5	16. 7	89. 5	9. 3
S-180 II	94. 2	65. 4	85. 0	59. 6
LLC-MK_2 (NCTC 3196)*	98. 7	17. 3	84. 5	13. 2

*Propagated in serum-containing medium.

was essentially the same as for HeLa 229, whereas the prefreeze plating efficiency of 53.8 percent was a characteristic of Detroit-6 and easily distinguished from that of HeLa 229. The two cell strains, however, were comparable in their response to the preservation procedures. With both strains the recovery of viable cells was uniformly high, and of the unstained cells recovered, the plating efficiencies were of the same order of magnitude as those determined before freezing.

In contrast, Detroit-504 differs from Detroit-6 and HeLa 229 in the following respects: Whereas both HeLa and Detroit-6 represent human epithelial-like, heteroploid cell strains, Detroit-504 is a human fibroblast-like, diploid cell strain. While cultures of Detroit-504 also consistently yielded harvests containing about 95 percent viable cells, including that of the prefreeze determination, the normal plating efficiency of this strain was considerably lower, and the plating efficiency values were not as reproducible as those obtained with the epithelial-like strains and fell between 5 and 17 percent. Nevertheless, the number of thawed viable cells recovered was about 90 percent of those frozen, which indicated that by this criterion only a small loss occurred. The plating efficiencies of the recovered viable cells were essentially the same as those frozen, which again indicated that regardless of the loss in viability, the proportion of unstained cells recovered had the same growth potential as those before freezing.

Preparations of strain S-180, originally derived from the solid transplantable mouse tumor (*14*), while containing viable cells to the same order of magnitude as the forementioned strains, had its own characteristic plating efficiency of about 65 percent. This strain responded differently to the freeze-preservation procedure in that a somewhat lower percentage of viable cells were recovered. However, of the unstained cells recovered, their plating characteristics were the same as the prefreeze cell suspension.

Similarly, a strain of cells derived from monkey kidney (*15*), while exhibiting a loss in viable cells of approximately 15 percent in the freeze-thaw procedure, again illustrated that the cells recovered retained their prefreeze plating characteristics.

It will be noted that the strains with the higher plating efficiencies exhibited more consistently reproducible values with respect to this determination, whereas those with lower plating efficiencies exhibited values within a somewhat broader range. Nevertheless, it was observed that no matter what loss in viable cells was incurred during the freezing procedure, their plating characteristics were retained. This would indicate that trypan blue staining might serve as a quantitative control of the preservation process, whereas plating efficiency might serve as a qualitative control of the possible selection of cells with regard to this one characteristic.

This is further illustrated by a change in the cell population occurring in one of these strains during serial propagation that was necessary to build up a seed stock containing sufficient numbers of cells for cell-culture collection. Table 4 gives the viable cell counts and plating efficiencies

TABLE 4.—Preservation of a population change occurring in Detroit-6 cells during passage

Passage history	Viable cells (% unstained)	Plating efficiencies (%)
Transfer 1	95. 0	55. 3
Transfer 2	96. 9	53. 8
Freeze 1	93. 7 Average	55. 0 Average
Transfers 3 to 5	94. 3	N.D.
Transfer 6	90. 7	52. 8
Transfer 7	98. 5	21. 1
Freeze 2	93. 6 Average	20. 5 Average

observed during serial transfer of the Detroit-6 strain. In this series of transfers, both the percent of viable cells and the plating efficiencies of harvests of the first 6 subcultures were consistent and representative of the strain, and preservation of the harvest of the second transfer exhibited characteristic cell-recovery data. During the seventh subculture, a marked change occurred in the strain—an alteration in the topographical appearance of the cell monolayers. Instead of the usual morphology of spread out, contiguous cells forming an epithelial-like layer, the cells were rounded, smaller, and inclined to form compact cell masses in which the cells tended to pile up. Concomitantly, a marked change in plating efficiency was observed in the harvest of the altered cultures, in that, in contrast to an average plating efficiency of 55 percent, the cells now exhibited a plating efficiency of 21 percent. The entire harvest was frozen at this point, and viability and plating-efficiency determinations were made before freezing and after thawing. The percentage of viable cells recovered was identical to previous experience with this strain in that it was uniformly high. Moreover, plating of the recovered cells indicated that in this characteristic the alteration occurring had also been retained in the freezing process. In addition, cells with the "high" plating-efficiency characteristic were still in the population (as demonstrated by subsequent plating experiments), which indicated that the freeze-thaw process did not select between the two morphologic types.

Of the various types of cell strains considered, it was interesting to examine one which possessed the nutritional characteristic of growth in completely synthetic media. Strain LLC-MK$_2$ (NCTC 3196), a clone derived from a monkey kidney strain (*16*) by Evans *et al.* (*15*), and adapted to growth in medium NCTC 109, was selected for this purpose. Initial attempts to preserve this strain in a serum-free "freeze" medium (NCTC 109 + 5% glycerol) resulted in poor and unpredictable recovery of cells from the frozen state. Moreover, we could not plate individual cells in the synthetic media. Therefore an experiment was conducted to study quantitative aspects of preservation in the presence of two concentrations of glycerol without serum, as compared to the usual freeze medium, and in which plating determinations were made with MEM plus 5 percent serum. (It was possible to propagate the strain in serum-free

MEM medium with results equivalent to NCTC 109.) These results are illustrated in table 5. In the absence of serum in the freeze medium only 55 percent of the frozen unstained cells could be recovered, irrespective of the amount of glycerol used, whereas in the presence of serum plus 5 percent glycerol 86 percent of the viable frozen cells were observed in the thawed preparation. Although the plating efficiencies were difficult to interpret in view of the low plating efficiencies obtained with the strain, it appeared the recovered cells did not have the same propensity for growth from the serum-free freeze medium as those frozen in the presence of serum. Although somewhat inconclusive, the combination of viability counts and plating efficiencies indicates that the optimal conditions for preservation of this strain have not been attained.

TABLE 5.—Effects of glycerol and serum on the "freezing efficiency" of LLC-MK_2 (NCTC 3196), a cell strain propagated in synthetic medium

	Prefreeze viability determinations		Cells recovered from storage	
Freeze medium	Viable cells (% unstained)	Plating efficiency* (%)	Viable cells (% unstained)	Plating efficiency* (%)
5% Glycerol + MEM	91. 6	14. 4	54. 6	2. 3
20% Glycerol + MEM	87. 8	13. 9	55. 7	2. 0
5% Glycerol, 15% calf serum + MEM	89. 7	14. 6	85. 8	6. 1

*Based on colony counts obtained from plating of cells in MEM-serum medium.

DISCUSSION

It is now well established that cultured cells can be readily preserved by procedures involving slow freezing in glycerol-serum media, storage at low temperatures, and recovery by rapid thawing. The rationale for these procedures has been discussed by Meryman elsewhere in this monograph. The methodology described herein differed from those previously reported for cultured cells only in certain technical details which were useful for adaptation of the procedures to the needs of the cell-culture collection. It was necessary to be able to preserve, from each strain, enough representative cell samples to meet the needs of characterization studies as well as to obtain a suitable quantity for a permanent repository. This required apparatus capable of readily handling a large number of vials under suitably controlled conditions. The controlled-rate slow-freeze apparatus, with liquid nitrogen for cooling, not only satisfied this requirement but also was useful to integrate the freezing phase of the process with the liquid-nitrogen storage phase. With regard to the latter, it is obvious that liquid nitrogen offers several advantages for the permanent storage of cultured cells. In addition to its ease of maintenance, the low temperatures that can be maintained are well below those at which damaging chemical or physical changes occur.

While various workers are in general agreement on the protection afforded by glycerol in the slow-freezing of cells, there is some disagreement on the establishment of an optimal glycerol concentration. The 20 to 40 percent glycerol concentrations used by earlier workers are definitely harmful to cultured cells. For example, we observed that with glycerol concentrations of 20 percent, or greater, recovery after thawing was quite unpredictable. Microscopic examination of such cells revealed large vacuoles, presumably of glycerol, within the cytoplasm, and these persisted for long periods during culture. Moreover, we have found marked evidence of cell damage in such cases, as shown by the bizarre morphologic appearance of the cells. In unpublished experiments (G. E. Foley, personal communication) it has been shown that glycerol is markedly inhibitory to cell growth in concentrations over 5 percent, as determined by methods employed for *in vitro* screening for cytotoxic or inhibitory compounds (*17*). The use of 5 percent glycerol in the freeze medium has the added advantage of not requiring its separate removal after thawing, if the cell density is such that the sample can be sufficiently diluted for culture. In addition to glycerol, serum also appears to have a protective effect during freezing. An example is demonstrated in one of the present experiments where it afforded unequivocal protection against loss of viable cells.

The establishment of optimal conditions for freeze-preservation of cultured cells, however, requires adequate criteria for judging their recovery from the frozen state. This problem has been approached qualitatively by such criteria as re-establishment of cultures from thawed cells or the ability of particular types of cells to retain certain characteristics after freezing, such as integrity of constituent chromosomes, tumorigenicity, metabolic activity, virus susceptibility, and cellular antigens. The most widely used quantitative procedure, however, has been the application of dye-exclusion tests, such as trypan blue staining, though there has been some question as to its significance as a suitable criterion of viability. Dye-exclusion tests would appear to determine only if a change (injury) has taken place in the permeability of the cell to the dye.

On the other hand, the experiments recorded herein suggest that a more meaningful interpretation may be made when the trypan blue test is carried out in relation to the ability of individual cells to attach to surfaces and multiply, *i.e.*, plating efficiency. As expected, when tested under the same cultural conditions, various strains exhibited individual characteristic plating efficiencies.

When subjected to the preservation procedure, some differences were observed between the various strains in the efficiency with which cells were recovered, as measured by trypan blue staining. It was significant, however, that the viable (unstained) cells that were recovered had the same plating efficiencies as those frozen. This can be interpreted, therefore, that even though cells may be lost in the freezing process, they are not lost selectively in their capability for attachment and growth. In such instances where a selective loss does appear, as observed in the recoveries of the monkey kidney strain adapted to growth on serum-free medium, it seems indicated that optimal conditions for cell preservation have not been attained. Thus, trypan blue staining and plating efficiencies combined might be considered a useful index for the quantitative and qualitative control of the cell-preservation procedure.

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DISCUSSION

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In our laboratory we have been freezing and storing tissue cells in vapor (−170° C) above liquid nitrogen. This has been a most effective method of preserving viability for over 2 years. Storage at −65° C, as recently reported by Ferguson (*1*), showed a significant decrease in the percentage of surviving cells over a period of 1 to 9 months. It is theoretically expected on physical principles that storage at −170 or −196° C will result in essentially indefinite preservation. There remains the task of experimental verification of preservation over very long periods at these temperatures.

In collaboration with Dr. G. F. Doebbler (*2*) of the Linde Company, we have been studying the effect of controlled rates of cooling on the preservation of tissue cells. Controlled constant rates of 1° C per minute to 45° C per minute over the temperature range from 0 to −50° C have been studied. Such cooling rates were automatically controlled by a differential thermocouple system, with one junction located at the specimen center and another in the chamber outside the vial. Cold or warm nitrogen gas was admitted to the cooling chamber through a solenoid valve to decrease the temperature at the desired rate. Viability was determined by the ability of decreasing dilutions of cells (120,000/ml to 7,500/ml) to attach to glass, extend their processes, and grow for 2 to 5 days. Vital stains were made with trypan blue, and metabolism was recorded by changes in *p*H of the medium at the end of 5 days. When cooling rates of 1° C per minute to 45° C per minute were used with HeLa and strain L cells frozen in 5 percent glycerol, we obtained almost equivalent growth of both cell lines at all rates. This finding is significant because it means one can freeze cells directly in vapor above boiling liquid nitrogen, a rate of cooling of approximately 20° C per minute. This provides a simple and convenient method of freezing tissue cells. Cell suspensions in glass vials may be placed directly in the vapor (−170° C) existing above the level of the liquid nitrogen (−196° C) in storage refrigerators. The cells can thus be frozen and stored in one operation, which obviates the need for expensive apparatus for the freezing operation itself. This method was effective for the preservation, in terms of growth, of 12 established cell lines and 4 primary cells that had not been previously cultured. At the present time, we cannot say what rate of cooling is ideal. This, of course, may vary with the cell lines and the particular cellular characteristics that are examined for preservation. When cooling rates above 45° C per minute were examined, a marked decrease in growth occurred as the cooling rate was increased to 18,000° C per minute. The freezing of cell suspensions in sealed vials is probably preferred, though we had no difficulties in *p*H change or other technical problems when cells were frozen in flint-glass, screw-cap vials, as long as they were not submerged in the liquid nitrogen.

I would like to comment on the use of additives. We have evaluated 6 additives of somewhat different types. Two were equally effective in the preservation of the viability, in terms of growth, of both strain L and HeLa cells. These additives were glycerol and dimethylsulfoxide (DMSO), used in 5 percent concentration. Sucrose and glucose were somewhat effective with HeLa, but not with the L cells. Methylcellulose and dextran, which do not enter the cell, were not effective. DMSO was reported more effective than glycerol in the preservation of bovine red blood cells by Lovelock (*3*) and of bone marrow cells by Ashwood-Smith (*4*). I would strongly suggest the trial of DMSO and possibly other additives in the freezing of tissue cells that have not been preserved well with glycerol. Since cells appear more permeable to DMSO than to glycerol, DMSO should also be tried with tissue explants, whose preservation poses a problem mentioned several times in this meeting.

In regard to the dye-exclusion test, as used by Dr. Stulberg, I would emphasize some caution in the use of vital staining as an indicator of viability. In our experience, staining with trypan blue does correlate to some degree with growth when freezing is done with glycerol at slow cooling rates. However, we have found two instances in which there is a lack of correlation: 1) when cooling rates greater than 45° C per minute are used, and 2) when certain other additives (methylcellulose and dextran) are added, even at slow cooling rates. In these instances, after freeze-thaw, unstained cells occur in numbers as high as 70 to 80 percent and yet no growth may be observed. Careful interpretation of these findings must be made, because such additives may sufficiently coat the surface of tissue cells, even after washing, to prevent the entrance of vital dyes. Because of the failure of protection of additives which do not enter the cell and the protection by several that do, it would appear that internal cellular damage is of critical importance. Recently, Weiss and Armstrong (*5*) presented evidence that much cytoplasmic damage is compatible with survival, but injury to the nuclear envelope or rearrangement of the nuclear contents are signs of irreversible damage.

In addition to the determination of viability in terms of growth, we must also look for the preservation of other cellular characters or functions—cellular antigens, enzyme production, hormone secretion, etc. The importance of such studies is demonstrated by the work of Morgan, Guerin, and Morton (*6*) which suggested that some isoantigens might be lost during freezing. In this connection, some of the techniques for detecting surface antigens (mixed agglutination) or internal antigens (gel precipitation) could be profitably used to evaluate the preservation of these cellular constituents.

In collaboration with Drs. N. R. Rose and T. K. Doebbler, we have also been interested in the production of antibodies by explants or suspensions of lymph-node or spleen cells obtained from immunized rabbits. We froze such cell suspensions in 5 percent glycerol or DMSO, thawed them, and either maintained or grew them *in vitro* with changes of medium every 3 to 4 days. The production of specific antibody (to bovine serum albumin) was detected in the medium by hemagglutination tests for 1 to 2 weeks. In these experiments, we were primarily interested in producing antibody and only secondarily in whether the cells actually grew or were simply maintained. I think the determination of growth rates and plating efficiency on frozen and thawed cells, as performed by Stulberg (*7*), Craven (*8*), and others, is the first major step in a reliable evaluation of viability. However, in the future, as we more critically study various methods of cell preservation for purposes of long-term storage, we will need to add to our criteria of viability the survival of specific cellular or metabolic characteristics.

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Detection and Elimination of Contaminating Organisms [1,2]

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LIKE DEATH and taxes, contamination or the threat of contamination is always with us.

Harrison's description in 1907 (*1*) of the formation of nerve fibers from cells of the frog neural tube suspended in a hanging drop of frog lymph opened a new field of cell biology. Obviously, contamination with bacteria, yeasts, and molds was the chief limiting factor in application of this new technique.

Technical improvements were contributed by Carrel (*2–4*), Maitland (*5*), Fisher (*6*), Maximow (*7*), Levi (*8*), Champy (*9*), Brachet (*10*), Strangeways (*11*), Gey (*12*), Lewis and Lewis (*13*), Earle (*14*), and Evans (*15*), which extended the uses of tissue culture and decreased the frequency of contamination. By ingenious techniques Carrel was able to keep connective tissue from the chick embryo in continuous culture for 34 years. However, many workers were discouraged by the high mortality of tissue culture through contamination. During the first 35 years after Harrison's original contribution, tissue culture remained the province of a small coterie of meticulous investigators who were not easily discouraged by contamination or willing to devise rigid aseptic techniques to prevent it. Most experiments during this period were short-term observations. They were on explants of tissue—in plasma clots or other biological culture media—which were difficult to reproduce or to prepare in large quantity or in identical replicate cultures for strict quantitative studies.

The transition from this period to the present affable state of cell culture was made possible by the incorporation of penicillin and other antibiotics in the culture medium (*16–19*). This simple step seemed to remove contaminants from the most grossly contaminated specimens. Additional impetus was given by: the cultivation of poliovirus in tissue culture by Enders, Weller, and Robbins (*20–22*), methods of replicate mass cultures (*23*), use of trypsin for preparing uniform cell suspensions (*24*), the production and successful use of the Salk poliomyelitis vaccine

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(*25*, *26*), the serial propagation of established cell lines (*27–29*), plaquing techniques (*30*), and single cell isolations (*31*). These achievements placed the art of cell culture within the reach of any investigator. Cell-culture techniques were adopted into biochemistry, physiology, embryology, virology, microbiology, pharmacology, pathology, and the disciplines of physics, biophysics, and mathematics. In return these disciplines have enriched the science of cell culture by contributing a continuous stream of innovations which extend the usefulness, reproducibility, and reliability of cell-culture methods. Time does not permit even an abbreviated listing of these contributions which are familiar to most of you. Instead I want to return to the subject of contamination because it is of vital concern to everyone using cell-culture technique.

In spite of the use of antibiotics, contamination of cell lines is widespread today, and one can guess that no laboratory is completely free of it. The contaminants include bacteria, yeasts, molds, viruses, protozoa, pleuropneumonia-like organisms (PPLO) or L forms, and contamination of one cell line with cells of another cell line. Antibiotics kill many contaminants but only suppress others and permit a chronic or latent infection which is not readily apparent. Penicillin is probably directly responsible for infection of cell cultures by L forms through selective pressure on chance bacterial contaminants (*29*). Indirectly, antibiotics are suspected of being responsible for the widespread cross contamination of cell lines through relaxation of strict aseptic techniques. Paradoxical as it may sound, removal of all antibiotics from tissue-culture media is in practice the best remedy for prevention of contamination of cell lines—it enforces meticulous aseptic technique, permits early recognition of contamination, permits use of antibiotics to treat an infected culture, minimizes development of L forms, and, through emphasis on good bacteriological technique, it is the best guarantee against cross contamination of cell lines.

BACTERIAL CONTAMINATION OF CELL-CULTURE LINES

To illustrate the problems caused by bacterial contamination of cell-culture lines, I will describe a single instance of such contamination in our laboratory and summarize what we learned from this and other similar occurrences.

Until 2½ years ago, we included penicillin, streptomycin, and mycostatin, in a concentration of 100, 100, and 25 μg per ml respectively, in all cell-culture nutrient media. Under such conditions most cultures remained sterile. However, the inapparent infection may invalidate a whole series of experiments. By inapparent, we mean that no gross or microscopic change can be detected in the tissue culture, and routine bacteriological cultures on blood-agar plates, media 199 and thioglycollate broth remain sterile. How then can the presence of infection be demonstrated?

In this experiment routine bacteriological cultures of Chang kidney cell line remained sterile, but cultures for PPLO regularly yielded a gram-positive diphtheroid. Culture for PPLO involved initial culture in PPLO broth with repeated subculture to solid media. The diphtheroid organism was readily isolated by the technique of repeated subculture in antibiotic-free media. The organism could also be demonstrated on primary culture if a centrifuged pellet of cells was used as inoculum. It could be seen as clusters of pleomorphic rods after painstaking search of stained monolayers of Chang kidney cells. Removal of all antibiotics from the tissue-culture media revealed the presence of the diphtheroid after one or two passages. The cells showed cytotoxic effects, the media became slightly turbid, and direct cultures on blood-agar plates were positive after 48 hours of incubation. An identical organism was recovered from 13 cell lines in our laboratory. Investigation showed that all these were being fed with calf serum from one supplier. Routine cultures of this serum on blood-agar plates had been sterile at 24 and 48 hours. However, subculture from broth to solid media of the 10-liter batch of calf serum on hand revealed the presence of the same diphtheroid.

Subsequent studies showed the following characteristics of this bacterial contaminant:

It failed to grow from infected tissue cultures containing antibiotics when inoculated into 199 broth without antibiotics, thioglycollate broth, or blood-agar plates. Cultures were readily obtained after subculturing to fresh plates or media 199 followed by an incubation period 48 hours or longer. The diptheroid did not grow within 48 hours when the calf serum was streaked on blood-agar plates, but did grow up within a week, forming a smooth colony with a pale-yellow pigment. It was a gram-positive, pleomorphic, nonsporing microaerophilic rod which was killed by heating at 55° C for 30 minutes. Incubation of the sealed vials of calf serum at 37° C for a week produced a faint ring of light-yellow pigment at the surface.

Positive cultures were also obtained from infected cell cultures by centrifugation and planting of the cell pellet directly on blood agar.

Microscopic examination of infected tissue cultures containing antibiotics demonstrated the presence of a few intracellular bacillary forms. Microscopy was not a reliable technique for detecting this bacterial contaminant.

In retrospect, our technicians observed that all 13 infected cell lines had been growing faster than usual for several weeks, necessitating the changing of media more frequently. During this time, the cell sheets tended to come loose from the glass container in large flakes within 4 days for the HeLa cells and after 1 week for most other cell lines. Subsequent studies proved that these changes were due to the presence of the bacterial contaminant and disappeared when the contaminant was eliminated.

To determine whether this antibiotic-resistant organism could be removed from the cell lines, we first titrated the maximal tolerated dose of several antibiotics which could be placed on the tissue cultures without killing the cells. An example is shown in table 1 for Chang's human kidney cell line.

TABLE 1.—Maximal tolerated concentration of antibiotic by Chang's kidney (μg/ml)*

Drug	Established cell sheets	Fresh transfers
Chloromycetin	32	8
Terramycin	64	32
Albamycin	128	64
Vancomycin	>512	>512
Erythromycin	>512	>512
Aureomycin	128	64

*Chang's human kidney cells grown in medium 199 plus 20 percent horse serum.

Next, the minimal inhibitory concentration of the antibiotics for the diphtheroid was determined as shown in table 2. These tests were done in the standard 199 broth with 20 percent horse serum. It will be noted that the diphtheroid was very sensitive to erythromycin and resistant to all others tested.

TABLE 2.—Inhibitory concentration of antibiotics for tissue-culture diphtheroid*

Drug	μg Per ml
Chloromycetin	500
Terramycin	500
Albamycin	125
Vancomycin	>1, 000
Erythromycin	0. 97
Aureomycin	1, 000

*Culture medium—199 medium with 20 percent horse serum.

All contaminated cell lines were treated for 6 serial passages by being grown in medium 199 plus 20 percent horse serum containing 200 μg per ml of erythromycin (Ilotycin Glucoheptonate—Lilly), 200 μg each of penicillin and streptomycin, and 25 μg per ml of mycostatin. All cultures remained free from this organism after treatment was discontinued.

To determine how long the contaminant had been present in tissue-culture cell lines and whether its presence had interfered with the antigenic comparison of cell lines we had been making for 2 years, we made studies which are shown in tables 3 and 4.

Table 3 shows the agglutination titer for the diphtheroid, of serums drawn from animals before and after immunization with various cell suspensions prepared from 3 to 8 months previously. There was no evidence that these animals had received injections of the diphtheroid in question.

Table 4 shows the complement-fixing activity of 4 normal serums when mixed with the diptheroid in question (#1 in table 4). These serums had all been used for feeding certain cell lines in the past. There was no

TABLE 3.—Agglutination titer for tissue-culture diptheroid of pre- and postimmunization serums from animals injected with various cell antigens

Antigen	Pre (A)	Post (D)
HeLa	80	80
Neuroblastoma	20	40
Wilms'	20	20
HeLa	10	10
Chang's kidney	20	40

evidence that the animals from which the serums were collected had developed antibodies to this diphtheroid.

Table 4 also shows the absence of CF antibody for the diphtheroid #1 in 16 antiserums prepared 2 months to 1 year previously against 12 cell lines and 4 animal serums. We therefore concluded that diphtheroid #1 was of relatively recent origin in our laboratory and had not interfered with the preparation of reagents for antigenic analysis of cell lines.

Diphtheroid #2 in table 4 was isolated from another subline of Chang's human kidney. This organism could not be eliminated by use of antibiotics. It fixed complement with low dilutions of antiserums prepared against Chang's kidney and conjunctiva, Salk monkey heart, and C-3 lines. This organism was apparently present in each line at the time cell antigens were prepared. Diphtheroid #3 was derived (by Mr. George Rothblatt) from a PPLO culture isolated from the same subline of Chang's kidney. As shown in table 4, it gave complement fixation with antiserums versus Chang's kidney and conjunctiva. This particular PPLO was possibly an

TABLE 4.—Complement-fixing antibody for tissue-culture diphtheroid in various tissue-culture reagents

Serum	Homologous titer	CF titer with diphtheroid #*		
		1	2	3
Normal horse	0	0	0	0
Normal calf	0	0	0	0
Normal human	0	0	0	0
Normal monkey	0	0	0	0
Antihorse	16	0	0	0
Anticalf	16	0	0	0
Antihuman	64	0	0	0
Antimonkey	64	0	0	0
Anti-ERK-D	—	0	0	0
Anti-ERK-D (calf-McLimans)	—	0	0	0
Antihuman intestine	256	0	0	0
Antimonkey kidney	8	0	0	0
Anti-HeLa	32	0	0	0
Anti-HeLa (calf-McLimans)	—	0	0	0
Anti-Chang's kidney	32	0	4	0
Antimonkey heart	512	0	0	0
Anti-Chang's conjunctiva	32	0	8	8
Anti-C_3	256	0	8	0
Anti-Chang's kidney	32	0	8	4
Antimonkey heart	256	0	16	0

*Diphtheroid #2 was isolated from another subline of Chang's kidney. Diphtheroid #1 is the organism described in this paper—it was found in 13 cell lines and in the calf serum used for nutrient media to feed the cultures. Diphtheroid #3 was derived by Mr. Rothblatt from a PPLO culture from Chang's kidney (personal communication).

L form derived from a diphtheroid in the presence of antibiotics. It had been present in Chang's kidney and conjunctiva when tissue-cell antigens were prepared for immunizing rabbits. Its presence, as well as diphtheroid #2, had interfered to some extent with antigenic analysis of the cell lines.

MOLDS

Infection of tissue cultures with molds occurs commonly from infected inoculums such as stool or saliva specimens or from airborne contamination in the laboratory. Molds tend to grow slowly and do not cause a serious problem for many short-term experiments. They can be detected by microscopic inspection of cell cultures and culture on special media. Treatment is usually unsuccessful, and it is wise to discard contaminated specimens—taking care not to open them in the laboratory and to make certain that they are autoclaved before being washed.

YEASTS

Yeasts are frequently present in clinical specimens submitted for viral diagnostic studies, in placental serum, and in dust. Some forms grow slowly in cell cultures and are not toxic for the cells. Other yeasts grow best in the presence of living cells and fail to grow on aerobic blood-agar plates. They can usually be detected by direct inspection, by removal of antibiotics from the cell-culture media, or by subculture on special media. Penicillin, streptomycin, and mycostatin are fairly effective in suppressing yeasts and molds in cell cultures, but when an infection is established it is usually resistant to treatment.

VIRUSES

Viruses are a common contaminant of cell cultures. In 1929 it was shown (*32*) that vaccinia virus could persist in tissue culture of immune cells, or multiply in roller-tube tissue cultures (*33*). Gey and Bang (*34, 35*) showed that several viruses could persist for many months in established cell cultures and Ackerman and Kurtz (*36*) demonstrated the persistence of poliovirus in HeLa cells. Rowe *et al.* (*37*) showed that primary cells from human adenoid tissue may harbor in a latent form many strains of adenovirus, as well as herpes hominiae, and cytomegalic inclusion virus. From primary monkey kidney cell cultures, Hull isolated many strains of adenovirus, vacuolating agent, and foamy agent virus (*38*).

Detection of virus in cell cultures is unsatisfactory in our present state of knowledge. If a distinctive cytotoxic effect is produced and recognized by the observer, the problem of detection is simple. If no typical effect is produced, subculture in other cell lines may reveal cytotoxic effects. Special stains may reveal inclusion bodies. Electron microscopy may be suggestive. The supernatant fluid from cultures may be tested for hemagglutination with human, guinea-pig, and chicken red blood cells. Hemadsorption tests on a monolayer of the cells may suggest the presence of a virus, as may interference with propagation of a known cytopathogenic virus. If none of these tests show the presence of virus, it is still not possible to be certain that a given cell line is free of virus or provirus—for which we have no method of recognition.

There is no sure method for eliminating an established virus infection from a cell-culture line. This is sometimes successful when cells are exposed to a combination of viral hyperimmune serum and terminal dilution techniques.

PROTOZOA

Free living amebae (*Acanthameba*) were found in primary monkey kidney by Jahnes, Fullmer, and Li in 1957 (*39*). These amebae grew well in HeLa, Chang's liver, human amnion, human skin, chick embryo, and rabbit kidney. We have studied these *Acanthamebae* and confirmed the high yield (10^5/ml) in tissue-culture fluid. Diagnosis of ameba infection of cell lines can be made by inspection or after hematoxylin-eosin stain but can be easily overlooked by the unwary. The cysts are stable for over 8 months in cold storage. *Endamoeba histolytica* also grows well in sterile monolayer cultures of HeLa and monkey kidney, in media 199 (*40*), or in Maitland-type chick-embryo tissue culture (*41*). We have on several occasions observed microfilaria in primary tissue cultures of monkey kidney. The microfilaria remained viable for 2 weeks and through 2 changes of nutrient media. Bland (*42*) grew toxoplasma in tissue culture, while others (*43*) have used tissue culture to study the protozoa of malaria, leishmania, trichomonas, and trypanosomes.

No spontaneous protozoan infestations have been reported in established cell lines, and their detection is more an oddity than a practical problem. However, tissue culture offers a good medium for investigation of protozoan infections (*43*).

PPLO AND L FORMS

Since the report of Robinson *et al.* (*44*) that PPLO were present in certain cell cultures, many observers have confirmed the high incidence of PPLO or L forms of bacteria in established cell lines (*45–49*). The recent report from Syverton's laboratory is the most extensive survey of cell lines (*50*). Of 166 sublines of 68 cell lines tested, 94 or 57 percent contained PPLO. These figures are in accord with previous reports. Detection of PPLO contamination is difficult by gross observation of the cell culture, but may be suspected if the cell sheet develops piled-up granular areas or tends to come loose from the glass after several days' incubation. Special stains may reveal intracellular PPLO (*51*) or fluorescent-antibody techniques (*52*) and electron microscopy (*53*) may be useful. However, the most reliable method at present is to culture the PPLO on such special media as the Difco PPLO broth and agar or fresh media, as described by Barile *et al.* (*54*) or Syverton (*50*). In our laboratory we have a higher percentage of recoveries by inoculating the cell-culture sediment into freshly heated PPLO broth in screw-top tubes, with subculture to freshly poured PPLO agar in sealed petri dishes after 4 and 8 days. Colonies are inspected at 100× and stained for confirmation (*55, 56*).

Several methods have been proposed for eliminating PPLO from cell cultures. Antibiotics such as terramycin, streptomycin, and neomycin tend only to suppress the infection (*57*), while penicillin promotes the production of L forms (*58*). Hyperthermia has been successful (*59*) in many cases—but in our hands has failed to remove PPLO from every infected cell culture. Apparently not all strains of PPLO are uniformly sensitive to hyperthermia to a degree which will permit survival of the tissue-culture cells. The most recent and most successful treatment is the use of kanamycin (*50, 53*) at a concentration of 100 μg per ml. Pollock *et al.* (*50*) found that 3 weeks of treatment was sufficient to remove PPLO from 15 cell lines with no apparent harmful effect on the cell lines and with no recurrence on removal of the kanamycin. In our own laboratory, 12 cell lines containing PPLO or L forms were treated with 100 μg per ml of kanamycin for 3 subcultures and then maintained without antibiotics. All have remained free of PPLO for 14 months. Others (*60*) have reported kanamycin-resistant strains of PPLO. Since kanamycin is so effective in eliminating PPLO or L forms from cell lines, it should not be included in the routine culture media and thus invite the development of kanamycin-resistant strains.

CELL CONTAMINATION

Contamination of cell lines with cells from another species was suggested by our comparison of cell antigens using the complement-fixation test (*61*). Rothfels *et al.* (*62*) confirmed this and showed by chromosome analysis that a culture of monkey kidney cells was contaminated by mouse L cells. From Syverton's laboratory (*63*) came final proof that cross contamination of established cell lines was not only possible but apparently a frequent occurrence in cell-culture laboratories. The results of chromosome analysis were in agreement with species red blood cell agglutination by guinea-pig serums prepared by injection of the whole cell culture. Of 28 established cell lines tested, 14 were contaminated by a cell from different species. Other techniques for determining the species of origin of a cell line have been developed and may prove superior to the guinea-pig immunization procedure. Stulberg *et al.* (*64*) have made rapid identification of cell lines by coating with species-specific fluorescent antibody.

Treatment of cell cross contamination is, of course, useless. One must place the emphasis on detection and prevention. Speculation about the method of contamination has not led to any convincing explanations, although Rothfels (*65*) has shown that recently established cell lines can develop marked alteration of karyotype in the absence of cross contamination. Gross errors of labeling or mixing pipettes are obvious mechanisms which most workers reject as unlikely to account for the frequent occurrence of contamination. We have demonstrated droplet infection by forcefully blowing HeLa or Detroit-6 cells from a pipette and catching the cells in a nearby open petri dish of culture media (fig. 1) containing a feeder layer of monkey cells. When subcultured 3 times the monkey kidney cells are almost completely replaced (fig. 2). In other tests, small droplet nuclei were produced by nebulizing a HeLa culture from a hand-operated De Vilbis atomizer. When the atomizer was held 1 foot over open petri dishes, viable cells were obtained, but so far we have been unable to recover these cells from the room air by drawing a small sample of air through tissue-culture media. Improved sampling techniques may eventually demonstrate that cells can survive for a time in dried, droplet nuclei.

How can one account for this widespread, multiple and diverse contamination of cell lines? Primary cell lines are much less frequently contaminated with PPLO or other cell lines than established cell lines (*52*) which are exposed to greater risk of contamination because of frequent transfers. Once a subtle contamination is established it tends to persist and may be mailed to other laboratories in exchange of cell cultures. The presence of antibiotics clearly masks or makes latent the presence of bacterial, yeast, and mold contamination, and penicillin definitely exerts selective pressure for the formation of stable L forms. All these observations suggest that much of the contamination is due to poor aseptic techniques in the laboratory, which develop under the false impression that the use of antibiotics will cover all sins.

To test this hypothesis we removed all antibiotics from culture media over 2½ years ago and instituted a regime of rigid aseptic technique. In theory this should: 1) enforce strict adherence to bacteriological principles of sterility and asepsis, 2) permit early detection of contamination when it does occur, 3) minimize the formation of stable L forms, and 4) reduce the possibility of cross contamination of cell lines—no matter what the mechanism of transmission.

The results have been gratifying indeed. Without antibiotics only 4 instances of bacterial contamination have occurred in the past 2 years. These were promptly recognized and discarded. No new PPLO or L form infections have been detected. Dr. Syverton's and Dr. Stulberg's laboratories have been following a similar regimen for a slightly shorter time with equally gratifying results. Our stock cell cultures are carried in milk-dilution bottles or roller tubes, but we have also grown batches of 40 Povitsky bottles without antibiotics. These experiences have led us to believe that eternal vigilance is the price of freedom from contamination.

Some of the technical methods which are helpful in maintaining an aseptic technique are as follows:

Tissue-Culture Laboratory

1. The room is provided with double doors and air lock to prevent drafts.
2. The doors are kept locked during working hours to prevent unauthorized personnel from entering.
3. The air-handling ducts throughout the laboratory are sprayed inside annually with propylene glycol containing 5 percent hexyl resorcinol. This removed dust from the air and makes it essentially sterile.
4. The walls and ceiling are painted with a bactericidal paint ("Wallshield" obtained from M. A. Bruder & Sons, Inc., 52d & Greys, Phila., Penna.).
5. Ultraviolet germicidal lamps (General Electric—15 watt Germicidal) are left on continuously. They are placed above eye level, and the beam is directed toward the ceiling.
6. All cells are passed in a 4 × 5 foot transfer room within the main tissue-culture laboratory.
7. A vacuum pump for aspirating culture media, operated by a foot switch, discharges outside the building. Fluid is drawn into a liter bottle containing Wescodyne (West Chemical Products Inc., 4216 West St., Long Island City, N.Y.) solution disinfectant. The bottle and tubing are washed and disinfected daily.
8. A walk-in 37° C incubator is provided in each tissue-culture laboratory so the technicians will not have to leave the laboratory during transfer operations.

Media

1. No antibiotics are used in media for stock cell lines.
2. Each line is carried in 2 separate media lots which are made with serum from different supply sources.
3. Media are made in a central kitchen and checked for sterility and retested for sterility by the technician who is planning to use the media.
4. Media are routinely tested on blood agar, Sabouraud's agar, nutrient broth, thioglycollate broth and M-yeast and mold broth (Baltimore Biological Laboratory, Baltimore, Md.). Cultures are held 1 week and the broth cultures are subcultured to blood agar.
5. Serum to be used in culture media is heated at 58 to 60° C for 30 minutes.

Operator

1. The technician wears a clean gown and a paper cap to cover the hair.
2. No talking is permitted in the transfer room. (If talking is necessary, a mask is worn.)
3. No unauthorized personnel are permitted in the laboratory.

Procedure

1. Wash table and floors with Wescodyne solution.
2. Leave cloth wet with Wescodyne on work table.
3. Repeat washing each time a new cell line or media is introduced.
4. Use screw-cap bottles or tubes for cultures whenever possible.
5. Use flame when cultures are opened.
6. Keep open cultures slanted.
7. Work rapidly in making transfers while container is open.
8. Keep tip of pipette below liquid when mixing.
9. Do not blow out last drop.
10. Place all glassware in steel pan under water and heat before washing.

11. Use burette with funnel for dispensing media.
12. Do all centrifuging outside the transfer room—in the main tissue-culture laboratory.
13. Transfer duplicate sets of stock cell cultures on separate days.
14. Test all cell lines for PPLO once a month—and for bacteria, yeasts, and molds, once a week.
15. Check cell and supernatant fluid for viruses—by hemagglutination of human, guinea-pig, and chicken red blood cells; inoculation into monkey kidney monolayer for cytopathogenic effect and by hemadsorption tests on growing monolayer of the cell line.
16. Store clean cell lines frozen in liquid nitrogen to avoid the risk of contamination by repeated serial passage.

SUMMARY

The use of antibiotics plus technical advances have permitted wide application of cell-culture techniques in all fields of medical research. Recent studies have shown that under the suppressing effect of antibiotics chronic contamination of cell cultures is common and difficult to detect. Contaminating organisms include bacteria, yeasts, molds, viruses, PPLO or L forms, protozoa, and cross contamination of cell lines. The frequency and diversity of contamination suggest that some drastic corrective measures are necessary. Removal of all antibiotics from stock cell cultures might enforce a return to strict aseptic techniques. Two years' experience has proved the feasibility of using modern cell-culture techniques without the use of antibiotics. The battle for freedom from contamination must be fought anew at each cell-culture passage, and, like political freedom, it cannot be assumed without effort—but must be defended at each succeeding generation.

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PLATE 1

FIGURE 1.—Three colonies of Detroit-6 cells growing on a monolayer of monkey kidney cells 24 hours after inoculation with approximately 1,000 Detroit-6 cells × 100

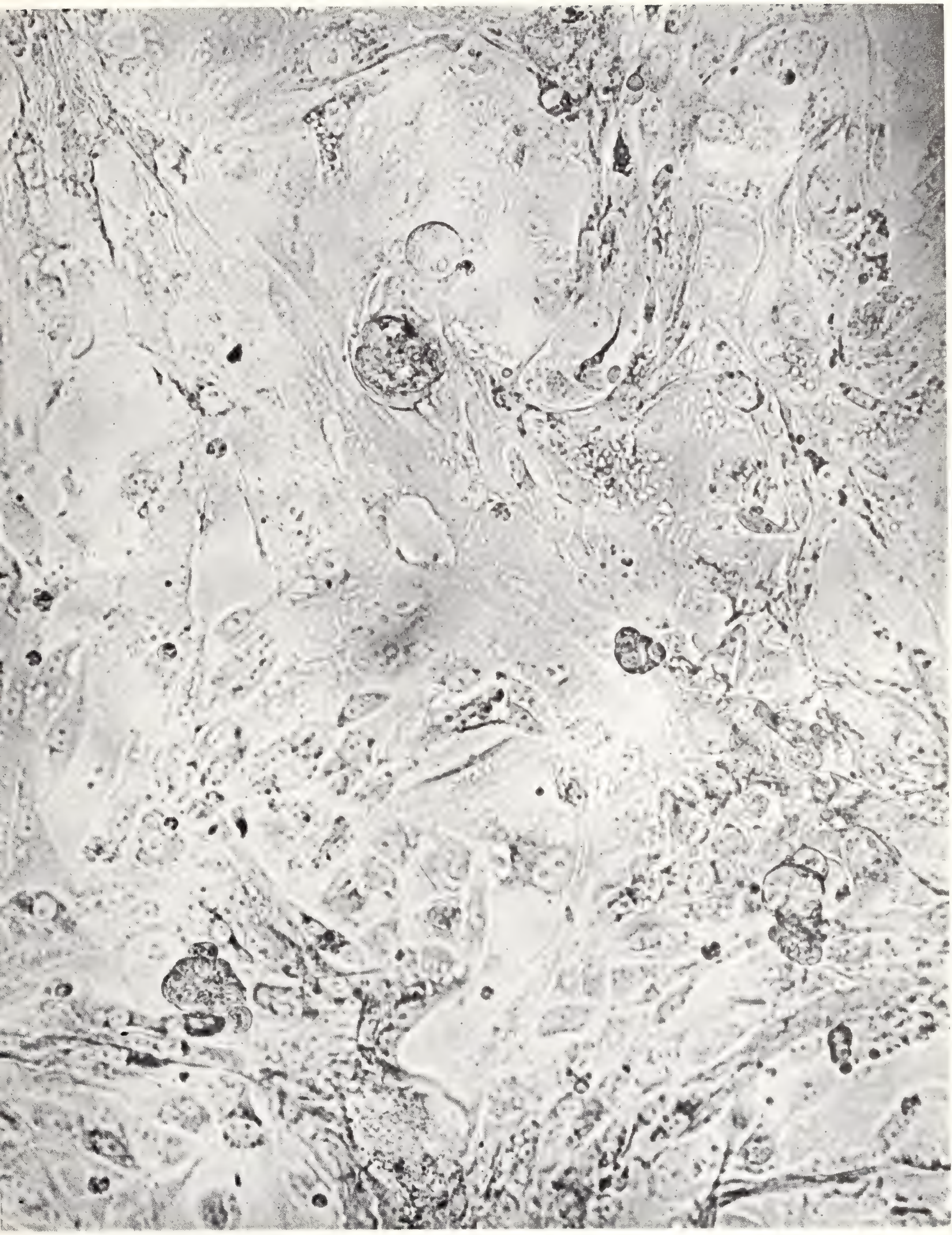

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Plate 2

Figure 2.—Same culture of monkey kidney-Detroit-6 cells as shown in figure 1, after subculture 3 times. There are only remnants and debris from the monkey kidney cells but many epithelial cells resembling Detroit-6.

CORIELL

DISCUSSION

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Dr. Coriell has delivered a thorough presentation of the over-all problems and solutions pertaining to detection and elimination of contamination in cell culture, to include such diversified groups as the viruses, pleuropneumonia-like organisms (PPLO), L forms of bacteria, bacteria, fungi, protozoa, and cross contamination with cell lines.

My comments shall be restricted to our observations with one of the typical and, perhaps, most common cell-culture contaminants, the PPLO. They will include the cultural procedures and fluorescent antibody technique we have developed for detection of PPLO and, also, our preliminary findings on current investigations for the elimination of PPLO: photodynamic inactivation and the use of PPLO antiserums as the serum component of tissue-culture media.

Several years ago we reported a medium for isolation and propagation of PPLO and/or L forms of bacteria (*1*). The basal media, now available commercially,[1] require only the addition of either blood or serums. Results obtained with this media for growth of tissue-culture PPLO strains, with blood or serums from various animal species under aerobic and anaerobic conditions, are shown in table 1. In brief, it was found that the addition of human, horse, and rabbit blood or serums promoted luxuriant growth of PPLO; whereas sheep blood and serum appeared to inhibit PPLO growth. Anaerobic conditions appeared superior to aerobic conditions in certain cases.

A survey was made comparing detection of PPLO by cultural methods with detection by the fluorescent-antibody technique. The results which have been reported shall be summarized here (*2*, *3*). A total of 102 cell cultures from 17 separate laboratories was examined. They were derived from at least 38 different primary and continuous cell types. Of the 102, 92 were continuous cell cultures representing 31 different cell types from 8 animal species, and 10 were primary cells representing 7 different cell types from 6 animal species.

It is shown in table 2 that PPLO were detected by the fluorescent-antibody technique in 49 out of 102 (48%) cell cultures. When the same cells were examined by the cultural procedures, PPLO were isolated from 48 of the 49 cell cultures found positive for PPLO by the fluorescent-antibody technique. The results obtained from the fluorescent-antibody technique and cultural procedures agreed in 101 of 102 (99%) cases.

The incidence of PPLO detection in relation to the nature of the cell cultures is shown in table 3. None of 10 primary cell cultures from 6 laboratories was positive for PPLO. Yet 48 of 92 (52%) continuous cell cultures from 13 laboratories were positive for PPLO. Ten of 15 laboratories using continuous cell lines (67%) submitted cells that were contaminated with PPLO.

Of continuous cell cultures propagated in media containing antibiotics, 46 of 64 (72%) were contaminated with PPLO, whereas only 2 of 28 (7%) cell cultures propagated in antibiotic-free media were contaminated with PPLO.

Cells grown in media containing serums had 48 of 86 (56%) contamination with PPLO, whereas none of 6 cultures from 3 laboratories in serum-free media had PPLO.

The incidence of PPLO detection in relation to the nature of the laboratories is shown in table 4. The activity of the laboratories could be grouped into 3 categories: 1) tissue-cell metabolic studies, 2) microbial propagation, and 3) tissue-cell suppliers. From 4 laboratories which used cells for metabolic studies, none of 31 cell cultures was positive for PPLO. From 9 laboratories which used tissue cultures for the propagation of microorganisms, 38 of 50 (76%) of the cells were contaminated with PPLO. From 2 tissue-cell producing laboratories 10 of 11 (91%) cell cultures examined were positive for PPLO.

We have found in this study that the incidence of PPLO contamination in cell cultures was *lowest* when 1) primary cells were used, and 2) antibiotics, and possibly serums, were *not* incorporated in tissue-culture media.

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[1] Designated as Bye agar and Bye broth for PPLO, Baltimore Biological Laboratory, Baltimore, Md.

TABLE 1.—Growth of tissue-culture PPLO strains on commercial PPLO media (Bye) with blood or serums from various animal species*

		Blood agar media						Serum broth media				
		Bye media					Brand X	Bye media				Brand X
Growth atmosphere		Human	Horse	Rabbit	Sheep	Human†		Human	Horse	Rabbit	Sheep	
Aerobic	Number positive / number tested	16/16	11/11	18/18	8/17	22/22	7/21	16/18	12/12	18/21	11/22	17/22
	Percent	100	100	100	47	100	33	89	100	86	50	77
Anaerobic	Number positive / number tested	17/17	12/12	20/20	16/20	22/22	17/22	16/20	12/12	22/22	20/21	22/22
	Percent	100	100	100	80	100	77	80	100	100	95	100

*Bye media with human blood was used for growth of 35 tissue-culture, 6 human, 10 avian, 5 murine, 1 bovine, and 1 saprophyte PPLO strain and numerous L forms of bacteria.

†Freshly prepared media (Barile *et al.*: Am. J. Clin. Path. 30, 1958).

Table 2.—Detection of PPLO in tissue-cell cultures

Procedure	Positive/No. examined	Percent positive
Fluorescent antibody	49/102	48
Agar culture	48/102	47

Table 3.—Incidence of PPLO detection in relation to the nature of the cell cultures

Nature	Source (number of laboratories)	Number positive / Number of cultures	Percent positive
Primary cells	6	0/10	0
Continuous cells	15	48/92	52
With antibiotics	13	46/64	72
Without antibiotics	3	2/28	7
With serums	13	48/86	56
Without serums	3	0/6	0

Table 4.—Incidence of PPLO detection in continuous cell cultures in relation to nature of laboratory work

Nature of laboratory work	Source (number of laboratories)	Number positive / Number of cultures	Percent positive
All laboratories	15	48/92	52
Physiological studies	4	0/31	0
Microbial propagation	9	38/50	76
Tissue-cell propagation	2	10/11	91

Based on our current data, it appears that tissue-culture PPLO strains have similar antigenic structures and that they differ from at least some human urethral, bovine, murine, and saprophytic PPLO strains (*3*). These findings do not support the view that the source of PPLO in contaminated cell cultures has a direct human origin.

Recent reports indicated that L forms of bacteria rather than PPLO are the responsible contaminants of cell cultures. If the organisms discussed in this paper are in fact L forms of bacteria, there is reason to suggest that a common bacterium was the source or that the L forms of at least some heterologous bacteria have common antigens.

Furthermore, if tissue-culture PPLO strains have a common antigen, then it may be possible to eliminate them from tissue culture by the use of antiserum as the serum component in the medium. Preliminary findings indicated that growth of different tissue-culture PPLO strains was inhibited in primary rabbit kidney cells in which PPLO antiserums had been incorporated in the medium.

In brief, we have found that media with 1 percent PPLO antiserums decreased the PPLO population but did not remove PPLO from cell cultures, whereas media with 10 percent antiserums removed PPLO completely within the second cell transfer. Although these findings have been encouraging, we would like to emphasize that these studies are in the preliminary stages.

Dr. C. W. Hiatt has been examining use of photodynamic inactivation as a means of eliminating simian viruses from cell cultures with good success (*4*).

Preliminary studies with Dr. C. W. Hiatt have indicated that PPLO are extremely sensitive to photodynamic action, and, for this reason, the procedure is currently under investigation as a means for elimination of PPLO from contaminated cell cultures. This procedure has the most potential advantage since it has been shown effective for a number of microorganisms, including certain animal (*4*) and bacterial (*5*) viruses, PPLO and/or L forms of bacteria, bacteria (*6*), fungi (*6*), and protozoa (*6*).

In conclusion, I would like to close with these remarks:

1) A number of good methods are available for the detection and elimination of PPLO from cell cultures. 2) Cell cultures should be routinely surveyed for PPLO contamination. 3) Investigators should know whether their cell cultures are contaminated, and, more important, whether the presence of PPLO in their cells influences the results of their investigations. 4) Cell cultures free from PPLO should be stored and available in case the working cell cultures become contaminated.

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Chromosomal Stabilization of Cell Strains[1]

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THE CHROMOSOMAL composition of normal tissues in primary cultures or during early periods of *in vitro* life seldom deviates from that of tissues *in vivo* containing predominantly euploid elements. If we examine the chromosomal composition of cells of many long-established strains, however, a drastic difference from the normal somatic constitution can immediately be noted.

To study the chromosomal and cellular changes in cells cultivated *in vitro* is both interesting and rewarding. Meaningful conclusions regarding the evolution of cell populations and experimental carcinogenesis have been drawn (*1*). An understanding of the nature of these changes may also help us to prevent their occurrence and thereby to stabilize the karyotype of cell strains. The main purpose of using tissue-culture techniques, of course, is to study cellular biology under controlled *in vitro* conditions. We obviously must be careful in extrapolating from results on aneuploid cells to *in vivo* diploid cells. It would be a great advantage if we had a method for growing stable cultures of diploid cells indefinitely. What are the patterns and nature of chromosomal changes in cells cultivated *in vitro*? Is it possible to initiate and maintain indefinitely a diploid mammalian cell line? Is there any way to minimize or to prevent chromosomal alterations in cell populations? Both the theoretical and practical aspects of these problems are considered in this brief review.

PATTERNS AND NATURE OF CHROMOSOMAL CHANGES IN CELLS CULTIVATED *IN VITRO*

Extensive heteropolidy is characteristic of many rapidly growing, continuous strains of human and animal cells of both normal and neoplastic origin (*2–14*). These cell strains usually consist of a mixture of cells with varying chromosome numbers and modified chromosome morphology, which are quite different from the diploid complement. In most strains, the majority of cells in the population is usually contained within a narrow range of connecting stemline chromosome numbers. Cells with chromosome numbers above or below the stemline zone are, however, not uncommon. Furthermore, the stemline number may shift under natural or artificial selection pressure. It is not surprising that a given strain under the same label may possess enormously dissimilar cytological constitutions at different times or under different culture conditions.

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[2] Operated by Union Carbide Corp. for the U.S. Atomic Energy Commission.

It is not my intention here to review the many instances reported in the literature of dramatic and permanent morphological, cytological, and functional changes that were later proved to be cellular contaminations. It suffices to point out that chromosome analysis, in conjunction with immunological, virological, and other techniques, has been found useful in the detection of contaminants (*11*).

A number of known cytological mechanisms can account for the formation of abnormal chromosome number and morphology in cell strains. First, a failure of cytokinesis or other processes, such as endoreduplication (*15*), may double the chromosome number of a cell. The resulting tetraploid cell, if it has no selective disadvantage, will persist in the cell population. Since it has a double quantity of genetic material, further chromosomal modifications can be better tolerated or even be selected. Levan and Biesele (*16*) found this pattern of change by following chronologically the cytological events in serial cultures of embryonic mouse-skin cells. The transformation process started with tetraploid formation, followed by a reshuffling of chromosome numbers, and ended with the establishment of predominating heteroploid stem lines. A similar pattern of population dynamics was observed by Hsu and coworkers in populations of the Novikoff rat hepatoma cells *in vitro* (*17*) and in a number of strains derived from tissues of C3H mouse (*18*).

Variation in chromosome numbers can occur at both the polyploid and the diploid level through mitotic irregularities such as nondisjunction. Cell strains can be established without going through the diploid-tetraploid-heteroploid sequence. Indeed, hyperdiploidy and hypodiploidy have been reported in cell strains derived from the Chinese hamster (*19–21*) and from the mouse (*12*), in clonal lines from the pig kidney strain PK-2 (*22*), and in a mouse tumor *in vitro* (*23*).

Finally, structural rearrangements of chromosomes also play an important part in the repatterning of karyotypes. New chromosomes atypical of the parental species arise in cell cultures through chromosome breakages and reunions. For example, metacentric chromosomes are found in cell strains from the mouse, which normally has 40 acrocentrics. New chromosome types such as minutes, extremely long chromosomes, rings, and dicentrics are frequently seen in established cell strains. Such changes, especially those involving the deletion of chromosomal material, are detrimental to cell survival at the diploid level, but conceivably have less effect at the polyploid level. These chromosomal mutations would provide raw materials for selection.

Apparently, cytological transformation represents a process of genetic readjustment of cells subjected to the *in vitro* environment, which is quite different from their original habitat *in vivo*. Moreover, conditions of *in vitro* growth may permit morphological changes to take place that are seldom or never expressed *in vivo*. Many environmental factors have been suggested as causal agents of transformation: heterologous culture medium, traces of toxic substances, latent virus, and background radiation. It is also true, however, that whatever is deficient or foreign in the medium may fail to provide an optimal growth condition for normal cells but may allow the accumulation of abnormal types.

Several years ago in our laboratory, pretested human serums from individual adult donors were used for maintaining diploid human cell lines (*24*), since it was found that individual batches of serum vary in the degree of toxicity to cells originating from different subjects. This problem of screening has been circumvented by the use of fetal human or bovine serum, though evidence of toxicity of certain batches of fetal bovine serum has also been reported (*25*). Preliminary experiments in Puck's laboratory (*25, 26*) indicate that the gamma globulin fraction of adult serum proteins contains factors toxic to cell growth *in vitro*. It was believed that the growth inhibitors present in adult serums (but not in fetal bovine serum) may be the major cause for the induction of heteroploidy. Using cultures of Chinese hamster cells from trypsinized lungs and kidneys, Ford and coworkers (*27*) compared the effects on chromosome stability of calf serum with fetal calf serum as a 20 percent supplement to medium NCTC 109. They found that fetal serum did not increase the chromosome stability though it did achieve a greater success in starting new cultures. Since the Chinese hamster cells rapidly undergo changes in culture, and since other culture conditions that obviously might also affect the chromosome stability were not defined in these experiments, these results cannot be considered as generally true.

Trypsinization, routinely used in monolayer cell cultures, is traumatic to cells (*28*) and may produce chromosome breaks (*16*). We have observed a higher frequency of chromosomal aberrations in cultures of mouse embryonic cells initiated by trypsinization than in those by the plasma-clot method. The chelating agent Versene (ethylenediamine tetraacetate), which is often used for cell dissociation, may produce similar effects.

The presence or absence of certain metabolites in the medium also may induce cellular alterations. Hsu and Manna (*29*) observed in two asparagine-nonrequiring cell lines of Jensen rat sarcoma that approximately 40 percent of cells showed one or more chromosome aberrations, including many complex chromatid exchanges, when the cells were grown in a medium lacking asparagine. The original Jensen sarcoma cells grown in primary cultures containing asparagine did not show any such abnormality. Metabolic wastes and cell disintegration products, including purines and pyrimidines, have been suggested as mutagenic agents (*30*). Both purine and purine riboside cause mitotic aberrations, including chromosome breaks (*31*); a thymine analogue, 5-bromodeoxyuridine, has been shown to accelerate polyploidization (*18*) and to cause chromosome breaks at specific loci (*32*).

It is clear that the factors causing heteroploid transformation and chromosomal damage are many and not completely known. However, a continued search will increase our ability to control these factors, and to slow down or prevent the process of karyotype alterations in cell populations.

CHROMOSOMAL STABILIZATION BY CLONAL ISOLATION

Establishment of clonal derivatives through single-cell isolation is probably one of the most effective methods of securing cell populations with relatively homogeneous genetic makeup. However, some clones may be more stable than others, and chromosomal fluctuations subsequent to the initial cloning usually take place (*9–11*, *27*, *33*, *34*). Although repeated cloning and frequent karyotype examination can insure the identity and genetic purity of a particular clone, experiments based on a derived heteroploid clonal cell line quite removed from the parental species have only limited biological significance. On the other hand, cloning would be a most useful technique to weed out the undesirable chromosomal variants from a predominantly diploid cell population, as in the case of a Chinese hamster diploid cell line (*35*), or to isolate genetic mutants in diploid cells, as has been attempted in heteroploid cell strains (*36–39*).

ESTABLISHMENT AND MAINTENANCE OF DIPLOID MAMMALIAN CELL LINES

Normal tissue explanted *in vitro* should, at least initially, give a true representation of the normal somatic chromosomes of the animal species in question. Attempts to maintain the normal karyotypes in human and animal cell lines, however, have met only with varying degrees of success. Normal diploid human cell lines from a variety of tissues have been developed and have retained growth activity and normal karyotype for different periods of time (*1*, *6*, *24–26*, *40*, *41*). Recently, Hayflick and Moorhead (*42*) reported they were able to maintain a number of human diploid cell lines of both embryonic and adult origin for as long as 50 subculture passages over a period of 1 year. In addition to karyotypic analysis, histology, virology, diagnosis for malignancy, and other criteria have been used to ascertain the normal characteristics of these cell lines. The cell lines survive, resume growth activities, and retain all characteristics after storage at −70° C for considerable lengths of time. The most significant finding is that despite the constancy of normal characteristics during the lifespan of the cell lines, none of them can escape eventual degeneration. Cold storage simply postpones this unavoidable fate. This phenomenon, according to Hayflick and Moorhead, may be attributable to intrinsic factors that are expressed as senescence at the cellular level. This gradual decline in growth rate, abnormalities in morphology, and eventual loss of human diploid cell lines after a period of apparent normalcy have been observed by Puck *et al.* (*25*) and in our laboratory. Meticulous care, as advocated on several occasions by Puck, can certainly prolong the lifespan of the diploid cell lines.

The findings among other mammals are variable. Kleinfeld and Melnick (*43*) observed a high frequency of cellular and mitotic abnormalties in short-term primary cultures of rhesus monkey kidney cells. In our experience with the chromosome cytology of primates (*44*, *45*), cultures derived from adult skin and kidney of a number of primate species, including the rhesus monkey, show remarkably stable diploid karyotypes for a period of several months. Hayflick and Moorhead (*46*) found that fetal rhesus monkey cells from many different organs retain diploidy until the 20th passage and are still doing well. Ruddle (*22*) found that the pig kidney strain he studied remained predominantly diploid for almost 5 years. Tjio and Puck (*41*) observed no appearance of deviation from the original chromosomal pattern in tissue-culture cells from the American oppossum, but found chromosomal irregularities in Chinese hamster cell cultures even during early *in vitro* life. Yerganian, Ford, and their coworkers (*19*, *20*, *27*) noted extensive karyotypic variations in cultures of Chinese hamster cells and were able to isolate by cloning a stable, actively proliferating diploid line (*35*). Stable diploidy can be maintained in rat cell cultures (*1*), but mouse cells begin to change their chromosomal constitution even in primary cultures (*16*, *18*, *47*, *48*).

Although species difference was suggested (*22*) as, and may indeed be, one of the factors influencing the relative stability of diploid cell lines, the variety of findings may simply reflect the inadequacy of our knowledge in growing cells of different origin. The successful development in recent years of certain diploid cell lines, in contrast to the seemingly inevitable heteroploid transformation of cultured cells described earlier, indicates a considerable technological advance.

The battle, however, in maintaining diploid lines is only half won. In primary cultures and relatively stable diploid cell lines occasional aneuploidy and polyploidy do exist. In addition, chromosomal aberrations of spontaneous nature occur in appreciable frequencies (*16*, *43*, *47*, *49–53*). Bender (*50*) maintained that, among diploid cells, those with fibroblast-like morphology show much higher spontaneous and induced chromosomal aberration frequencies than do those with epithelial-like morphology. Sax and Passano (*52*) further noted that the spontaneous chromosomal aberrations in a diploid fibroblast-like human cell line, scored as post-metaphase bridges and fragments, increase with the age of the cell line.

Almost all the spontaneous chromosomal aberrations detected are of the chromatid type. Terminal deletions and certain types of exchanges may be deleterious to the cell, and those possessing such aberrations are automatically eliminated from the culture. However, cells of other aberrant types, including aneuploid or polyploid cells, may survive and continue to propagate within the population. Furthermore, cryptostructural alterations of chromosomes and point mutations that remain undetected cytologically must also be accumulating in the course of long-term cultivation.

The problem of accumulation in pure cultures of naturally occurring genetic variants confronts all biologists alike. Microbiologists have long practiced type-collection and characterization of established cultures. Similar measures have now been taken for mammalian cell strains and have been discussed in this symposium. The karyotype, the analysis of which has been made possible for mammalian cells by cytological advances in recent years, is one of the important criteria for cell identification and for detection of certain abnormalities.

SUMMARY

Recent developments have made it feasible for a prolonged maintenance of actively growing, nearly uniform and reasonably stable, euploid mammalian, including human, cell lines. Although the availability of diploid cell lines is a definite improvement over the use of mixed, undefined populations of heteroploid cells, karyotypic abnormalities exist even in diploid cell cultures. Certain practical measures, such as prevention of cross-contamination, rigid control of culture conditions, and repeated clonal isolations should help to preserve the genetic purity of the cell line. Additionally, a repository of frozen stock cultures can serve not only as controls but also as sources of supply of uniform material adequate for many types of biological experiments. Diploid cell lines, unlike heteroploid and even malignant cell strains, probably cannot be propagated perpetually. However, reisolation of tissues from the same individual or from inbred animals should overcome this difficulty.

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DISCUSSION

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Dr. Chu's elegant and succinct presentation of the current status of our knowledge of chromosomal stability in tissue cultures has as its consequence an implication of very great importance. The implication is that with the possibility of maintaining mammalian cells in serial culture with retention of the diploid configuration, a major achievement results which permits new uses for cell cultures for very practical purposes. The use of human diploid cell strains could result in their use: 1) for the production of human virus vaccines (as already achieved in our laboratories with the Koprowski attenuated poliovirus strains, which are now undergoing a limited field trial, 2) as a tool for the exploration of the possible existence of human tumor viruses, 3) as controls in screening potential cancer chemotherapeutic compounds *in vitro*, 4) in establishment of cell banks containing functional human cells, and 5) in studies of "normal" human cells *in vitro*.

The use of diploid cell strains in contrast to the heteroploid cell lines is analagous to the choice one might make in using inbred versus randombred animals in his experimental designs.

As Dr. Chu has pointed out, Dr. Moorhead and I have been active in this area for some time and have at present accumulated no less than 50 diploid cell strains, predominantly from human fetal skin and muscle, lung, liver, and kidney (*1*). Some strains have been derived from human adult skin. A consideration of the major characteristics of heteroploid cell lines, of which over 200 have been reported in the literature (*2*), makes the inclusion of the diploid cell cultures under the term "cell line" inapplicable (table 1).

A definition of terms is therefore a necessary consideration. Precedence and usage confine the term "cell line" to only those cells that have been grown for years *in vitro*. Thus a presumption of potential immortality is made for the "cell line." Diploid human cells lack this character. In addition, all mammalian cell lines examined to date vary from the diploid number. This fact alone should exclude the diploid cells from being termed "cell lines," therefore we have chosen to refer to them

TABLE 1.—Differential characteristics for human cell lines and cell strains

Character	Cell lines	Cell strains
Chromosome number	Heteroploid	Diploid
Sex chromatin	Not retained or variable	Retained
Histotypical differentiation	Not retained	Partially retained
Growth in suspended culture	Generally successful	Unsuccessful
Pathological criteria for malignancy as determined on biopsies of cells inoculated into hamsters or human terminal cancer patients	Positive	Negative
Limitation of cell multiplication (life of strain or line)	Unlimited	Limited
Virus spectrum compared to corresponding primary tissue	Often different	Same
Cell morphology compared to corresponding primary tissue	Characteristically different	Same
Acid production	Less than that produced by equal number of cell strain cells	More than that produced by equal number of cell line cells
Retention of Coxsackie A-9 receptor substance	Lost	Retained
Ease of establishment	Difficult (not predictable)	Usually successful

as "cell strains." Before the discovery of cell lines, populations of cells grown for limited periods *in vitro* were referred to as cell strains. Therefore it seems desirable to maintain this consistency in nomenclature and avoid the confusion of recent years wherein these two terms have been used interchangeably. Shifts in chromosome number from the diploid configuration probably are a necessary condition or consequence of the establishment of a "cell line" with the resultant acquisition of unlimited growth potential. Since we have also derived strains of human cells from mongoloid individuals which contain 47 chromosomes, and from others with 45 chromosomes, we have proposed the following definition of terms:

A *cell strain* is a population of cells derived from animal tissue, subcultivated more than once *in vitro*, and lacking the property of indefinite serial passage while preserving the chromosomal karyotype characterizing the tissue of origin.

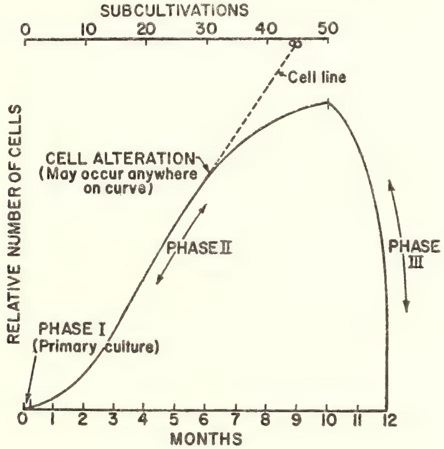
A *cell line*, conversely, is a population of cells derived from animal tissue and grown *in vitro* by serial subcultivations for indefinite periods with a departure from the chromosome number of the tissue characterizing its source. It is also possible to include in this definition, when observed, the characteristic of cell "alteration" as described by Parker, Castor, and McCulloch (*3*), and by Hayflick (*4*). It is proposed that the term "cell transformation," which has been used interchangeably with "cell alteration" (*5*), be excluded because the latter term has historical precedence and "transformation" has specific implications in the allied field of bacteriology which do not, as yet, apply to cell culture. The terms "established cell lines" and "stable cell lines" should, likewise, be avoided, as the former is redundant and the latter implies that changes have ceased to take place or are no longer possible.

Primary cells should indicate those cells obtained from the original animal tissue that have been cultivated *in vitro* for the first time. If subsequent passages of these cells are made, presumably such cells can properly be called a *cell strain* until they either are lost through further subcultivations or alter to the heteroploid state, in which case they could properly be referred to as a *cell line*.

The diploid strains that we have investigated are fibroblast cultures when derived from human fetal organs and adult skin (figs. 1–4). We have also been able to isolate strains from fetal rhesus monkey organs. So far only the kidney and lung have been examined karyologically. Both strains are apparently diploid at least up to the 40th passage and have been carried serially to date for 8 months and over 50 subcultivations. The unusual characteristic of all the monkey strains is that they are composed of epithelial cells, in contrast to the fibroblast strains, which are always derived from corresponding organs of human material that we have examined. The diploid monkey kidney strain supports the growth of many human viruses including poliovirus, and its utilization for human vaccine production becomes apparent. Carrying such strains for at least 20 subcultivations, with a 2:1 split ratio, means that from a very small piece of monkey kidney one can derive at least a million 1-liter bottles of diploid monkey kidney cells for possible vaccine production. We have not encountered any simian viruses in this fetal strain.

Other characteristics of the diploid human strains include the fact that they cannot be subcultivated, in general, beyond about 50 passages, at which time they cease to divide. The reason for the eventual death of the strains does not appear to implicate media components, pleuropneumonia-like organisms (PPLO), latent viruses, or the depletion of some nonreplicating component of the primary cell population. The explanation may be found in the possibility that some cumulative effect takes place that is expressed perhaps as aging or senescence at the cellular level. This may be explained by postulating a factor, necessary for cell survival, whose rate of duplication is less than that of the cell (asynchrony). It is possible to conceive of two separate self-duplicating systems, one of which (the hypothetical) is contained in the other (the cell). A slight reduction in replication rate of the hypothetical system could possibly lead to a gradual depletion of this factor to a critical or threshold level within 50 generations. Conversely, the rate of synthesis itself may be unchanged, but a slightly higher rate of loss (through some unknown environmental condition) would eventually yield the same result. This may be interpreted as a cumulative effect. A parallel situation exists in biology in those species of paramecia containing the Kappa factor, whose rate of multiplication can be influenced environmentally without influencing the rate of cell multiplication.

This concept appears to explain best that in our experience and those of others (*6, 7*), no one has succeeded in carrying diploid human cells for periods much over 1 year. It is not implied that any exact number of passages are required or that a definite period of time is necessary to predispose the cells to death, but simply that a finite limit to the cultivation of human diploid cell strains does exist. Text-figure 1 diagrams our concepts concerning the history of cell lines and cell strains *in vitro*.



TEXT-FIGURE 1.—Diagrammatic representation of the history of cell strains and the phenomenon of cell alteration. Phase I, or the primary culture, terminates with the formation of the first confluent sheet. Phase II is characterized by luxuriant growth necessitating many subcultivations. Cells in this phase are termed "cell strains." An alteration may occur at any time giving rise to a "cell line" whose potential life is infinite. Conversely, cell strains characteristically enter Phase III and are lost after a finite period of time. This scheme is applicable, in a general way, to most animal cells grown *in vitro*.

The diploid cell strains are initiated and subcultivated under conditions that are identical to the handling of cell lines. In contrast to earlier reports (*6, 8*), no unusual methodology is employed. Although the strains are eventually lost after about 50 passages, storage of surplus cultures from any or all passages at low temperatures implies that any given strain will always be available, for all practical purposes, since the cell yield from 50 passages is about 2×10^7 metric tons. Sex chromatin of female strains is always retained. The cells also retain a primitive degree of histotypical organization in which multilayered membranes are formed with the concomitant production of a thick intercellular matrix which is presumably collagen (figs. 5–7). Unlike heteroploid cell lines, the diploid strains will not multiply in suspended culture, nor will they produce tumors when inoculated into the hamster cheek pouch by the method of Handler and Foley (*9, 10*) or when inoculated subcutaneously into terminal human cancer patients according to the procedure of Southam, Moore, and Rhoads (*11*). The virus spectrum of the human strains is very broad, and 31 different human viruses, so far examined, can be grown in them (table 2).

This information which we have accumulated concerning diploid human cells, plus the work of others as presented by Dr. Chu, is offered in the hope that it will stimulate the interest of those who can foresee the potential value of this type of system.

Table 2.—Virus susceptibility of human diploid cell strains

Cell strain	Virus	Cytopathogenic effect
WI-1 (Passages 20–35)	Measles	+
	Coe	+
	Adenovirus type 2	+
	Adenovirus type 12	+
	Coxsackie A-9*	+
	Coxsackie A-13	+
	ECHO-9	+
	Reovirus type 1*	+
	Poliovirus Koprowski-Chat type 1 (attenuated)	+
	Poliovirus strain TN type 2 (attenuated)	+
	Poliovirus Koprowski-Fox type 3 (attenuated)	+
	Poliovirus type 1 (Mahoney)	+
	ECHO-21	+
	Varicella†	+
	Rabies (strain CVS)	Virus growth; variable CPE
	Mengo	+
	Herpes simplex	+
	Endomyocarditis virus (EMC)	+
	Influenza (type A Jap)	+
	Influenza (type B Lee)	+
	Influenza (type C Colindale)	—
	Vaccinia	+
	Yellow Fever (strain 17D)	+
	Coxsackie B-1	—
	Coxsackie A-1	—
	Western equine encephalitis	—
	Polyoma	—
WI-10 (Passages 17–25)	ECHO-11*	+
	ECHO-20*	+
	ECHO-22*	+
	ECHO-28*	+
	Respiratory syncytial (strain Long)*	+
	HE virus (Pett. strain)*	+
	Salisbury (strain H.G.P.)*	+
	Salisbury (strain F.E.B.)*	+
	Parainfluenza 1*‡	—
	Parainfluenza 2*‡	—
	Parainfluenza 3*‡	+

*Tested by Drs. Vincent V. Hamparian, Albert Ketler, and Maurice R. Hilleman of the Division of Virus and Tissue Culture Research, Merck Institute for Therapeutic Research, West Point, Penna.

†Tested by Dr. Eugene Rosanoff, Wyeth Laboratories, Radnor, Penna.

‡Detected by hemadsorption.

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PLATES

PLATE 3

FIGURE 1.—Strain WI-1. Diploid human fetal lung cells after 35 subcultivations and 9 months *in vitro*. Phase contrast. × 360

FIGURE 2.—Strain WI-1. Diploid human fetal lung cells after 35 subcultivations and 9 months *in vitro*. Stained with May-Grünwald-Giemsa. × 300

FIGURE 3.—Strain WI-1. Directional orientation of cells after 3 days in culture of the 35th passage. Cell orientation presumed due to phenomenon of contact inhibition. Stained with May-Grünwald-Giemsa. × 100

FIGURE 4.—Strain WI-1. Colony development from single cells planted in a petri dish. Photographed after 35 days *in vitro* of 36th passage; 50 mm petri dish.

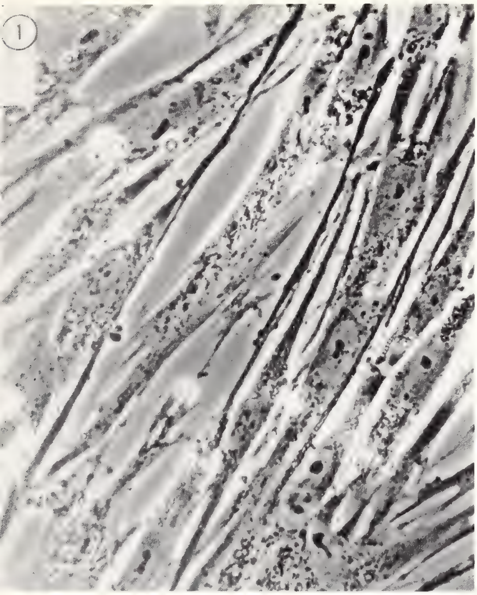
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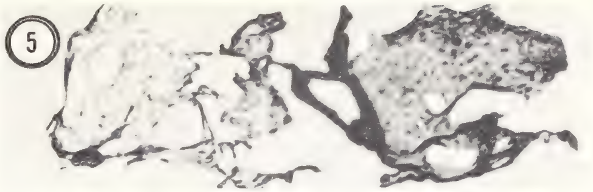
Plate 4

Figure 5.—Strain WI-1, 36th passage. Membrane spread out on surface of a glass slide, fixed and stained with May-Grünwald-Giemsa. × 1.1

Figure 6.—Strain WI-1, 36th passage. Microscopic appearance of a membrane. *Note* multilayer cellular organization and intercellular matrix. Hematoxylin and eosin. × 103

Figure 7.—Same as figure 6. Higher-power magnification. × 470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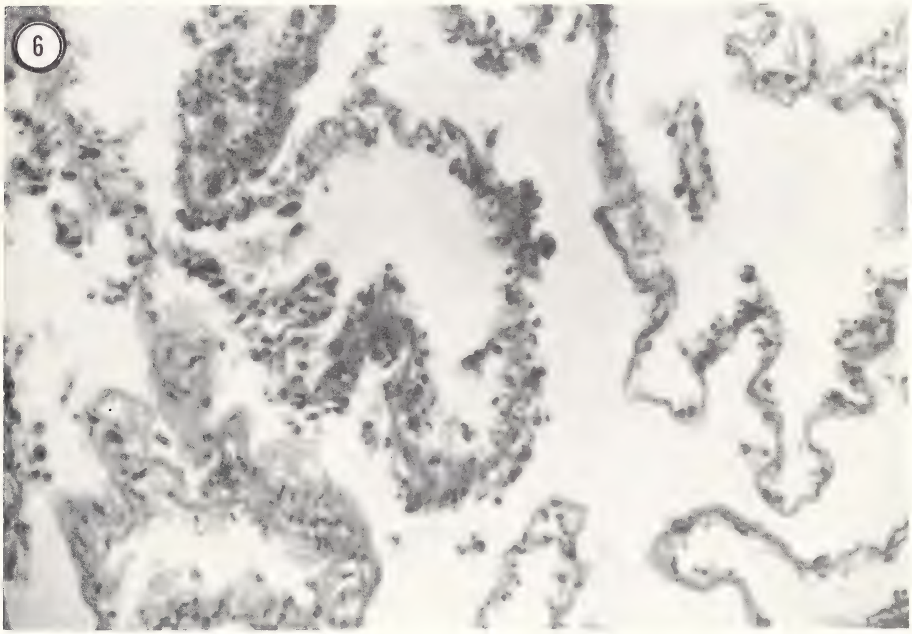
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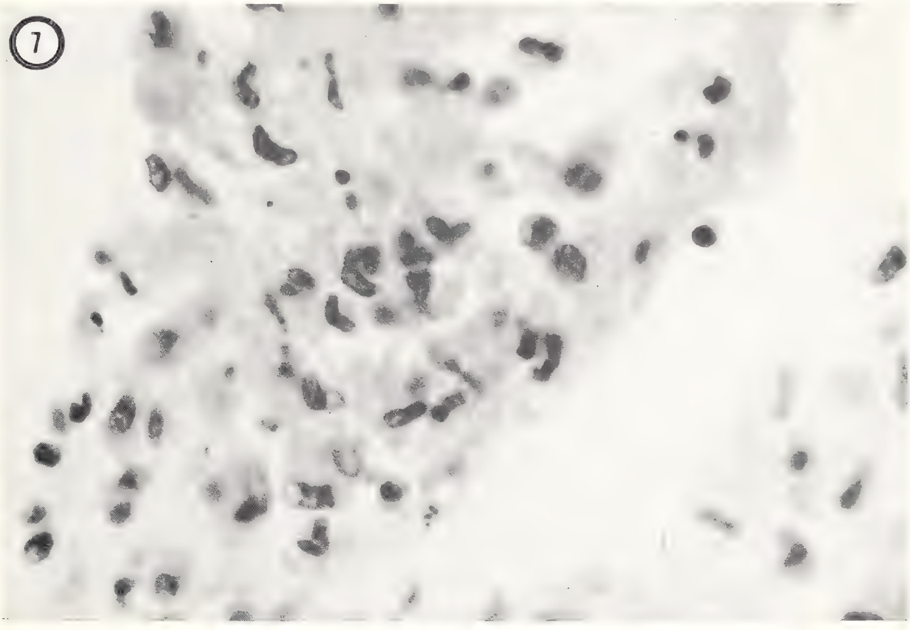
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Session II

Characterization of Cell Strains

Chairman: T. C. Hsu, *Ph.D., University of Texas M.D. Anderson Hospital and Tumor Institute, Houston, Texas*

Genetic Studies With Human Cell Lines [1,2]

WACLAW SZYBALSKI, ELIZABETH HUNTER SZYBALSKA, *and* GIORGIO RAGNI,[3,4] *McArdle Memorial Laboratory, University of Wisconsin, Madison, Wisconsin*

IN 1958–59 we reported on our first attempt to develop a usable system of markers for quantitative genetic studies employing human cell cultures (*1–3*). Two sequential mutant lines were isolated, the first D98/AG, with approximately 100-fold increased resistance to 8-azaguanine (AG) but retaining sensitivity to 8-azaguanosine (AGR), and the second D98/AGR, with 60-fold higher resistance to AGR. The mutation rates from the parental line D98S to D98/AG and from D98/AG to D98/AGR were determined (*3*), and the effect of ultraviolet light (UV) on one of these mutational events was examined (*2, 4*). Since that time this mutational system has been further extended and refined, the enzymatic basis of the mutant phenotypes elucidated (*5*), and the system evaluated for a variety of genetic applications, including mutagenicity and DNA-mediated transformation studies.

MATERIALS AND METHODS

From the epithelial-like human cell line, Detroit-98, the clonal line D98S was derived by single-cell isolation (*2*). This line was selected on the basis of its favorable cultural characteristics, hardiness, constant chromosomal number (63, as determined by cooperative study with Dr. A. Levan), and ability to form compact colonies with high plating efficiency. The cell culture procedures and quantitative methods for estimation of cell survival used in the present studies were described in the previous publications (*2, 6*). The most commonly employed growth medium (E90) was Eagle's basal medium supplemented with 10 percent horse serum.

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[1] Presented at the Syverton Memorial Symposium on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

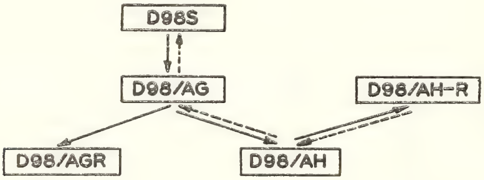
[2] This study was made under grants CY-3492 and CY-5215 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] Fellow of the Public Health Service, National Institutes of Health, Bethesda, Maryland. *Present address:* Pierrel s.p.a., via Appia, Capua (Caserta), Italy.

[4] The able technical assistance of Miss Sandra Joannes is gratefully acknowledged.

DEVELOPMENT OF GENETICALLY MARKED STRAINS

The development of genetically marked cell strains is a prerequisite for quantitative genetic studies. Resistance to purine analogs or their ribosides provided suitable markers, since it was relatively easy to select single-step mutants exhibiting normal plating efficiency and growth rate at analog concentrations many times higher than the inhibitory threshold for the sensitive parental line. Text-figure 1 summarizes the types of mutations under present study.



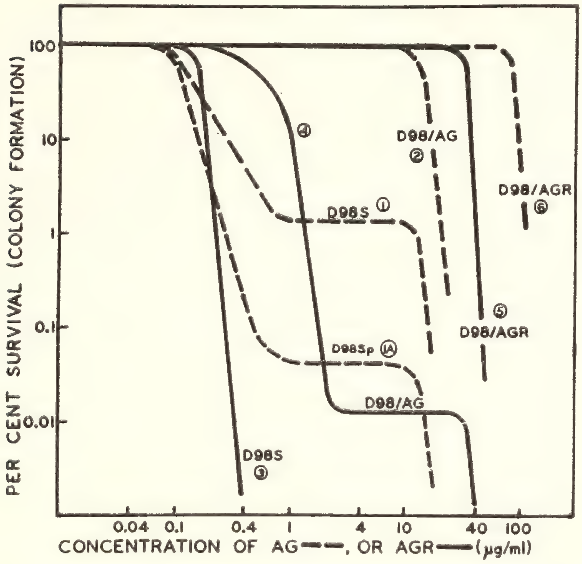
TEXT-FIGURE 1.—Origin of mutants sequentially derived from line D98S. Properties of mutants are summarized in tables 1 and 2 and in text-figures 2 and 3.

8-Azaguanine-Resistant Mutants

When D98S cells were plated and grown at increasing concentrations of AG, the number of cells giving rise to colonies was unaffected up to a level of about 0.1 μg AG per ml. Above this concentration, there was a sharp drop in plating efficiency, and then a leveling off at approximately 1 percent of the colony-forming cells in the control (or 0.02% for the "purified" line D98Sp—*cf.* text-fig. 2 and the paragraph on "Mutant frequency in purified lines"), with a plateau extending from 1 to 10 μg AG per ml, followed by a second precipitous drop. The line D98/AG was developed from a clone growing at 6 μg AG per ml and found to be a stable, highly resistant mutant exhibiting high plating efficiency at AG concentrations up to about 10 μg per ml. Curves 1 (1A) and 2 (text-fig. 2) represent the survival of strains D98S (D98Sp) and D98/AG in the presence of increasing concentrations of AG.

8-Azaguanosine-Resistant Mutants

The 100-fold increase in resistance to AG in the mutant line D98/AG is accompanied only by an approximately threefold rise in resistance to AGR. Curves 3 and 4 (text-fig. 2) represent the respective survivals of lines D98S and D98/AG in the presence of increasing concentrations of AGR. One may see that 0.01 percent of the D98/AG cells are able to form colonies at AGR concentrations of 3 to 30 μg per ml. By isolation of these survivors, a stable mutant line D98/AGR was established, which plates with high efficiency at AGR concentrations up to 60 and 200 times higher than the inhibitory thresholds for the D98/AG and D98S lines,



Text-figure 2.—Survival, as measured by colony formation, of cloned human cell line D98S, its subline D98Sp "purified" on HAT medium (*see* p. 78), and of two mutant lines D98/AG and D98/AGR in the presence of increasing concentrations of 8-azaguanine (AG) and 8-azaguanosine (AGR). Reproduced in part from the earlier publication by Szybalski (*3*).

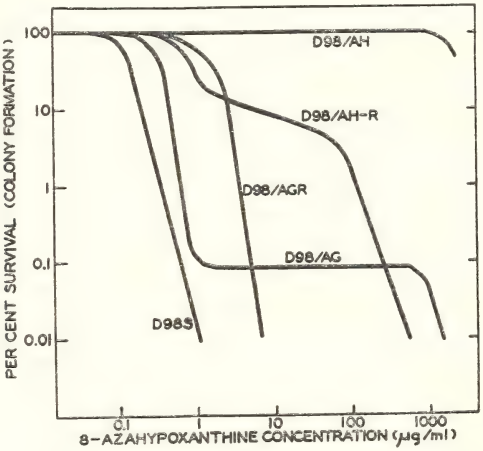
respectively. Curves 5 and 6 (text-fig. 2) represent the survival of the D98/AGR mutant line at increasing concentrations of AGR and AG, respectively.

8-Azahypoxanthine-Resistant Mutants

All three lines, D98S, D98/AG, and D98/AGR, are inhibited by another purine analog, 8-azahypoxanthine (AH), at concentrations above 0.1 μg per ml (D98S), 0.2 μg per ml (D98/AG), or 1 μg per ml (D98/AGR). When one of the sublines of D98/AG was plated at increasing concentrations of AH, a survival curve similar to that plotted for D98S on AG was obtained (text-fig. 3). The plating efficiency was constant at about 0.1 percent over the concentration range of 2 to 500 μg AH per ml, corresponding to the plateau of the curve. One of these resistant clones gave rise to the mutant line D98/AH, which proved to be 7,000 times more resistant to AH than its parental stock, D98/AG, and has retained this resistance over a period of over a year during subculture in AH-free medium. The survival of these lines in graded concentrations of AH is represented in text-figure 3.

8-Azahypoxanthine-Sensitive "Revertants"

The acquisition of resistance to AH, a hypoxanthine analog, in the D98/AH mutant line is accompanied by loss of the capacity to utilize the natural metabolite hypoxanthine as sole purine source. In the usual medium (E90) all the cell lines can synthesize *de novo* the necessary purines, and thus addition of preformed purines is superfluous. However, in the presence of inhibitory levels of aminopterin, a folic acid analog which blocks folic acid reductase (*7*), the pathway of *de novo* purine synthesis is disrupted together with other single-carbon-transfer reactions leading to the synthesis of thymidylate and certain amino acids. The inhibition of thymidylate synthesis can be circumvented by adding thymidine to the medium (*8*), which already contains sufficient amounts of the required amino acids. Thus, in the presence of aminopterin and supplemental thymidine, *de novo* purine synthesis is *selectively* inhibited, and the cells are able to grow only if a utilizable preformed purine source is supplied (*cf.* text-fig. 4). A medium containing 0.02 μg aminopterin per ml (20 times the 50% inhibitory level), 5 μg thymidine per ml, and 5 μg hypoxanthine per ml of E90 medium (HAT medium) permits normal growth of all the AH-sensitive lines. D98/AH cells, on the other hand, are completely inhibited, being unable to utilize hypoxanthine. The HAT medium thus provides selective conditions for screening for hypoxanthine-utilizing revertants from D98/AH.



TEXT-FIGURE 3.—Survival of human cell line D98S and of four mutant lines, the derivation of which is shown in text-figure 1, in the presence of increasing concentrations of 8-azahypoxanthine (AH).

When the standard D98/AH line, after being carried for a number of months, was plated on HAT medium, approximately 0.1 percent of the cells gave rise to colonies, which upon isolation were found to be AH-sensitive clones able to utilize hypoxanthine as sole purine source (*9*). Survival of this "revertant" line, designated D98/AH-R, in graded concentrations of AH, represented in text-figure 3, is characterized by a gradual decrease in the plating efficiency, as indicated by the slope of the curve. The interpretation of this behavior and other properties of the D98/AH-R line will be discussed in following sections.

Utilization of Preformed Purines

The capacities of the cell lines to utilize preformed purines were compared, as a prerequisite for (*i*) studies on the biochemical basis of resistance, and (*ii*) the development of conditions for selection of analog-sensitive cells and for purification of the sensitive lines. The aminopterin-thymidine medium, described previously, satisfying the conditions of selective *de novo* purine synthesis inhibition was supplemented singly with adenine, hypoxanthine, guanine, their ribosides, and some nucleotides in graded concentrations. The relative capacities of the cell lines to utilize these metabolites, as sole purine sources, expressed as the concentration of purine or riboside permitting 50 percent of the cells (as compared with the control) to form colonies on the aminopterin-thymidine medium, are given in table 1. The results can be summarized as follows:

TABLE 1.—Relative capacities of mutant cell lines to utilize preformed purines and purine ribosides during *de novo* purine synthesis inhibition

Purine source	Concentration permitting 50 percent survival* on AT medium† (μg/ml)				
	D98S	D98/AG	D98/AGR	D98/AH	D98/AH-R
Adenine	0. 06	0. 12	0. 23	0. 04	0. 08
Adenosine	0. 13	0. 28	0. 33	0. 10	0. 25
Hypoxanthine	0. 08	0. 5	0. 9	>500	0. 09
Inosine	0. 12	0. 9	0. 7	200	0. 16
Inosinic acid	0. 23	1. 1		700	0. 26
Guanine (5μg/ml)	4. 9%‡	0. 9%‡	0‡	0‡	0‡
Guanosine (10 μg/ml)	8. 3%‡	2. 8%‡	0‡	0‡	1. 1%‡
Guanylic acid (12 μg/ml)	3. 0%‡	4. 5%‡	0‡	0‡	1. 1%‡

*Colony formation, as percent of control.

† E90 medium containing 0.02 μg per ml aminopterin and 5 μg per ml thymidine.

‡Percent survival at the indicated concentrations; higher survival levels not obtainable with increasing concentrations of guanine, guanosine, or guanylic acid.

(*a*) Adenine and adenosine serve as sole purine sources for all the cell lines and are utilized somewhat less efficiently by D98/AG and D98/AGR than by the other lines.

(*b*) Guanine, its nucleoside and nucleotide, only partially support the growth of D98S and D98/AG cells, under these conditions, while no colony formation was observed with the other lines.

(*c*) Hypoxanthine and its riboside (inosine) are utilized by the D98S, D98/AG, and D98/AGR lines, but 5 to 10 times higher concentrations are necessary to permit normal growth of the AG- and AGR-resistant cells when compared with the sensitive D98S cells.

(*d*) The D98/AH line does not utilize hypoxanthine even at 500 μg per ml, while inosine (or inosinic acid) is utilized only at 1,500 times higher levels than those required by D98S.

(*e*) The D98/AH-R "revertant" line has regained the ability to utilize hypoxanthine at low concentrations.

Selective Removal of Pre-existing Mutants

Mutational studies would be greatly facilitated if conditions could be devised which permit selective elimination of the background of pre-existing mutants under study. The systems described here fulfill this experimental requirement. The D98S line was purified of AG-resistant mutants by growing it on aminopterin and thymidine-containing medium supplemented with a limiting concentration of hypoxanthine (0.15 μg per ml) (S-HAT medium), conditions which permit the growth of the parental strain while selectively inhibiting the mutants because of their lower capacity to utilize hypoxanthine (table 1). By consulting table 1, it is possible to design a variety of conditions for selective elimination of purine analog-resistant mutants from the sensitive populations.

The reciprocal task, the elimination of pre-existing sensitive revertants in the resistant populations, can be achieved by subculturing the latter at purine analog concentrations selectively toxic to the sensitive revertants, as determined from table 2. Thus the existence of both preformed and *de novo* pathways of purine nucleotide synthesis provides effective means for (*i*) the selection of *both sensitive* and resistant mutants and (*ii*) the reciprocal elimination of pre-existing mutants.

Sensitivity and Cross Resistance to Other Purine Analogs

The pattern of resistance and cross resistance to other purine and purine riboside analogs was determined for all the cell lines, as a further aid in elucidating the biochemical basis of resistance and in devising suitable selective systems. The inhibitory levels of various analogs, expressed as that concentration permitting 50 percent survival (colony formation, as % of the control), are given in table 2. The results can be summarized as follows:

(*a*) Most of the purine analogs can be grouped into four broad classes:
  (A) adenine analogs, as exemplified by 8- or 2-azaadenine, 2,6-diaminopurine, and 2-fluoroadenine, all highly active inhibitors;
  (B) hypoxanthine and guanine analogs, *e.g.*, 8-azahypoxanthine, 6-mercaptopurine, 8-azaguanine, and 6-thioguanine, all highly active inhibitors;
  (C) xanthine analogs, *e.g.*, 8-azaxanthine, with little or no inhibitory activity;
  (D) other inactive purine analogs, not included in table 2.

(*b*) There is no substantial degree of cross resistance between classes *A* and *B*, but very pronounced cross resistance within class *B*.

(*c*) Although the cross resistance within class *B* is quite pronounced, the individual analogs differ from each other in their patterns of inhibition. The first mutational step, to AG resistance (D98/AG), appears to be associated with a greater degree of cross resistance to the guanine analogs (AG and 6-thioguanine) than to the hypoxanthine analogs (AH and 6-mercaptopurine). The second mutational step, to AH resistance (D98/AH), augments predominantly resistance to the hypoxanthine analogs, although the over-all change in sensitivity for the D98S to D98/AH transition seems to be most pronounced for AH (16,000×) and approximately equal for the other hypoxanthine and guanine analogs (2,000–3,600×). The cross-resistance patterns for the riboside analogs seem to be more complex.

Biochemical Basis for Mutant Phenotypes

Two types of biochemical experiments, performed in cooperation with Dr. R. W. Brockman of the Southern Research Institute, were summarized earlier (*5*), and will be described elsewhere in detail. The conversion of

TABLE 2.—Sensitivity and cross resistance of mutant cell lines to analogs of purines and purine ribosides

		Sensitivity* (μg/ml)	Factor of cross resistance†			
		D98S	D98/AG	D98/AGR	D98/AH	D98/AH-R
	Inhibitor		D98S	D98/AG	D98/AG	D98/AG
Adenine analogs	8-Azaadenine	0. 6	1. 3×	1. 5×	1. 0×	0. 19×
	2-Azaadenine‡	0. 10	2. 3×	2. 2×	0. 3×	
	Purine	0. 10	1. 5×	1. 7×	1. 3×	
	2,6-Diaminopurine	0. 40	2. 0×	1. 3×	0. 5×	0. 25×
	2-Fluoroadenine§	0. 002	1. 3×	1. 3×	0. 5×	
	8-Azaadenosine¶	0. 004	2. 3×	2. 8×	0. 9×	0. 11×
	2-Fluoroadenosine§	0. 005	2. 0×	2. 0×	0. 3×	
Guanine and hypoxanthine analogs	8-Azaguanine (AG)	0. 12	100 ×	7 ×	33 ×	0. 42×
	6-Thioguanine‖	0. 006	20 ×	3. 3×	100 ×	70 ×
	8-Azahypoxanthine (AH)	0. 12	2. 3×	3. 9×	7000 ×	2. 1 ×
	6-Mercaptopurine‖	0. 06	3. 0×	18 ×	1200 ×	0. 6 ×
	8-Azaguanosine (AGR)¶	0. 15	3. 3×	60 ×	40 ×	4. 4 ×
	6-Thioguanosine**	0. 018	12 ×	2. 3×	120 ×	60 ×
	8-Azainosine††,¶	0. 10	1. 0×	5 ×	5 ×	2. 3 ×
	6-Mercaptopurine riboside§	0. 14	4. 3×	8 ×	120 ×	0. 22×
	8-Azaguanylic acid**	0. 10	2. 0×	80 ×		22 ×
Xanthine analogs	8-Azaxanthine‡‡	100	No inhibition at 100 μg per ml			
	8-Azaxanthosine¶	50	1. 0×	2. 0×	0. 5×	

*Concentration permitting 50 percent survival (colony formation, as % of control).

† Ratio of sensitivity of mutant strain (indicated in numerator) to sensitivity of strain D98S or D98/AG (indicated in denominator).

‡Obtained through the courtesy of Dr. G. B. Brown, Sloan-Kettering Institute.

§Obtained through the courtesy of Drs. J. A. Montgomery, H. E. Skipper, and W. E. Fitzgibbon, Jr., Southern Research Institute.

‖Obtained through the courtesy of Dr. G. H. Hitchings, Burroughs Wellcome & Co.

¶Obtained through the courtesy of Dr. J. Davoll, Parke-Davis & Co., Ltd.

**Obtained through the courtesy of Drs. R. B. Ross and H. Bond, Cancer Chemotherapy National Service Center, National Institutes of Health.

††Obtained by chemical deamination of 8-azaadenosine; may be contaminated with traces of the latter.

‡‡Obtained through the courtesy of Dr. G. Levin, The Hebrew University-Hadassah Medical School, Jerusalem.

C^{14}-labeled purines or their analogs into the corresponding nucleotides by either intact cells or 5-phosphoribosyl pyrophosphate-supplemented cell-free extracts was measured.

(*a*) All the cell lines exhibited approximately equal adenylic acid pyrophosphorylase activity.

(*b*) The D98S and D98/AG lines were characterized by approximately equal guanylic and inosinic acid (IMP) pyrophosphorylase activities, while D98/AGR preparations displayed about one half and D98/AH-R less than one twentieth of this activity; D98/AH did not exhibit any measurable pyrophosphorylase activity with radioactive hypoxanthine, guanine, azaguanine, or 6-mercaptopurine as substrate.

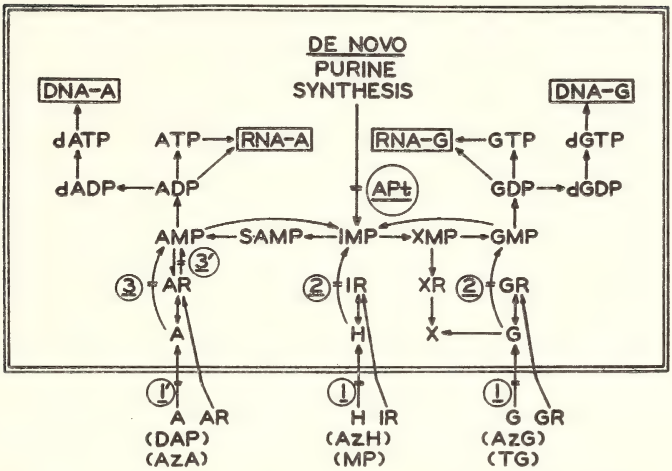
(*c*) Intact D98S cells actively converted C^{14}-guanine and less efficiently C^{14}-azaguanine into the corresponding nucleotides. This conversion was reduced by a factor of 10 in the D98/AG cells and was barely detectable for the D98/AGR and D98/AH lines. All three lines, D98S, D98/AG, and D98/AGR, were approximately equal in converting hypoxanthine to inosinic acid, while the D98/AH line showed only one twentieth of this activity.

(*d*) All the lines actively deaminated guanine and 8-azaguanine to xanthine and 8-azaxanthine, respectively.

These data indicate that the first mutational step, D98S to D98/AG, is *not* associated with a significant decrease in nucleotide pyrophosphorylase activity, but probably with impairment of some reaction prior to the base-to-nucleotide conversion (text-fig. 4). Preliminary experiments on short-term exposure of intact cells to C^{14}-azaguanine, followed by centrifugation in hematocrit tubes, indicated that the transport of AG into D98/AG cells takes place at about half the rate observed with D98S cells. This block, observed with free-base analogs but much less with their ribosides, augmented by the efficient deamination of AG, could conceivably explain the high AG resistance of D98/AG.

The high resistance of the D98/AH mutant to all the hypoxanthine-guanine analogs and its inability to utilize hypoxanthine and inosine are in agreement with the observed complete loss of measurable IMP pyrophosphorylase activity (*cf.* text-fig. 4). This function is partially regained in the D98/AH-R "revertant," possibly due to a suppressor-type mutation rather than a true reverse mutation at the same site, with resulting very gradual drop in survival at increasing concentrations of AH (text-fig. 3). This pattern is consistent with the concept of a very low IMP pyrophosphorylase activity unevenly distributed among the cells or selectively repressed by higher concentrations of AH. Whether these mutations are associated with actual loss or only with modification of the enzyme could be determined only by quantitative immunochemical studies on the IMP pyrophosphorylases from the mutant cells.

The D98/AGR mutant is also resistant to 8-azaguanylic acid (AGRP), in agreement with the general observation that nucleotides are not able to penetrate intact cells without previous dephosphorylation. On the other hand, AGRP seems to be consistently endowed with activity at least twice that of AGR, when the molar inhibitory concentrations against three of the mutant strains are compared (table 2).



TEXT-FIGURE 4.—Idealized and abbreviated diagrammatic representation of the *de novo* and *preformed* pathways of purine metabolism in D98 mutant cell lines. A, H, X, G: adenine, hypoxanthine, xanthine, guanine; R: ribosides; MP, DP, TP: mono-, di-, triphosphates; IR: inosine; S-AMP: adenylosuccinic acid; d: deoxynucleotides; *1*, *1'*: block in uptake of H, G, 8-azaH (AzH), 8-azaG (AzG), 6-thioG (TG), 6-mercaptopurine (MP), A, 8-azaA (AzA), 2,6-diaminopurine (DAP), prior to ribophosphorylation; *2*: block in ribophosphorylation of H, G, and their analogs; *3*, *3'*: partial or complete block in ribophosphorylation of A and its analogs, and in AMP kinase activity, respectively; APt: aminopterin-imposed block of *de novo* pathway of inosinic acid biosynthesis (HAT medium). Mutation from D98S to D98/AG corresponds to blocks *1* and *1'*; from D98/AG to D98/AGR to partial block of *2*; from D98/AG to D98/AH to complete block of *2*; from D98/AH to D98/AH-R to partial restoration of pathway 2. Reproduced from Szybalski and Szybalska (*10*).

GENETIC APPLICATIONS

Determination of Mutation Rates

Until rather recently, there were no data available on the rates of somatic mutations in human cells. The determination of the mutation rate from AG sensitivity to resistance, using a "clonal variance test," was described by Szybalski (*3*). A still simpler method, the principle of which was summarized by Szybalska and Szybalski (*9*), and which is better adapted to the measurement of very rare mutations, will be described here in more detail.

A. Clonal variance test—This method is based on the variance test described for bacteria by Luria and Delbrück (*11*) with modifications by Newcombe (*12*) and Ryan *et al.* (*13*). The tissue-culture method, in which each glass-attached colony is considered equivalent to one test-tube culture of the Luria-Delbrück variance test (*11*), is applicable only to moderately high mutation rates and was described in detail elsewhere (*3*). With this method, the mutation rate from D98S to D98/AG was determined to be in the neighborhood of 5 × 10^{-4} mutations per cell per generation and from D98/AG to D98/AGR approximately 1 × 10^{-6} mutations per cell per generation.

B. Mutant frequency in purified lines—This method takes advantage of the principle outlined for the elimination of pre-existing mutant cells. Under these conditions the mutation rate can be derived directly from the frequency of newly arising mutants: To measure the mutation rate from AG sensitivity (D98S) to AG resistance (D98/AG), D98S cells were first "purified" by subculturing in S-HAT medium (0.02 μg aminopterin, 2 μg thymidine, and 0.15 μg hypoxanthine per ml E90 medium), and later plated (at 50,000 cells per dish) in a selective medium containing 6 μg AG/ per ml, supplemented for the first 2 days with 2 μg thymidine per ml. The purpose of the thymidine supplement was to prevent "thymidine-less death" in resistant cells recovering slowly from aminopterin inhibition. The frequency of AG-resistant mutants, which was normally 0.01 to 0.02, and sometimes as high as 0.2 in certain D98S sublines, dropped sharply in the S-HAT medium and after 2 weeks of growth reached a constant level of about 2×10^{-4}. This corresponds to a mutation rate of approximately 3×10^{-4} ($2 \times 10^{-4} \times \sqrt{2}$) (*13*), which differs only by a factor of less than 2 from the rate of 5×10^{-4} mutations per cell per generation determined earlier by the "clonal variance test" (*3*). Curve 1 (text-fig. 2) represents the survival of the original D98S line and curve 1A the corresponding survival of the D98S subline obtained from D98S by 2 weeks' growth in the S-HAT medium.

Applying the same principle, pre-existing AH-resistant mutants can be eliminated from the D98/AG line by subcultivation of the latter in the highly selective HAT medium, which completely inhibits the IMP-pyrophosphorylase-lacking D98/AH cells. Purification of the D98/AG line by continued cultivation under these selective conditions resulted in a drop in the AH-resistant mutant frequency to a level of the order of 4×10^{-6}. Further quantitative studies with this mutational system are in progress.

The method based on elimination of pre-existing mutants is not so well adapted to the measurement of mutation rate from AH resistance to the partial regain of IMP-pyrophosphorylase function (D98/AH to D98/AH-R) because of the capacity of the "revertant" line for limited survival at high AH concentrations (*cf.* text-fig. 3). Thus continued cultivation of the D98/AH line in the presence of 100 μg AH per ml resulted in only a tenfold drop in the frequency of "revertants." On the other hand, a series of single-clone isolates of the D98/AH line formed no "revertant" colonies when inocula of 10^5 cells were plated on HAT medium, indicating that the "reversion" must be relatively rare. Further quantitative studies on this mutational system are also in progress.

Mutagenicity Studies

Our first attempt (*4*) to test the effect of UV light on mutation toward AG resistance yielded a paradoxical result, namely, that the proportion of mutants actually decreased, while reconstruction experiments (*2*, and unpublished) showed that the D98/AG mutants are actually more resistant to UV. Since this and other early experiments on chemical mutagenicity were undertaken before the idea of elimination of pre-existing resistant mutants by subculturing on S-HAT or other selective media was developed, the mutagenicity studies are at present undergoing experimental re-evaluation and will be only very tentatively discussed here. The effect of many known mutagens on the D98Sp to D98/AG mutational process is being determined. Before plating on the AG-containing selective medium, the D98Sp cells are treated with putative mutagens in one of several ways, including: (*a*) 4-day growth in the presence of relatively nontoxic analogs, *e.g.*, 5 chloro- or 5 bromodeoxyuridine, which are incorporated into the nucleic acid component of the cells (*6*); (*b*) overnight growth in the presence of subinhibitory concentrations of several toxic agents; and (*c*) 2-hour exposure to alkylating agents or other noxious chemicals, including nitrogen mustards, triethylenemelamine, and β-propiolactone, at concentrations resulting in 10 to 80 percent cell survival. A variable period of growth on AG-free medium, prior to addition of selective concentrations of AG, was tried to permit the phenotypic expression of the AG resistance character. To date no clear-cut mutagenic effect has been observed with the system under study.

DNA-Mediated Genetic Transformation

Up to this time well-documented cases of DNA-mediated genetic transformation have been reported only for bacteria. On the other hand, the infectivity of viral RNA and DNA for mammalian cells has often been described. It has also been shown in several recent publications that high-molecular DNA can be incorporated into mammalian cells.

It was therefore interesting to test whether any transforming activity could be detected for isolated mammalian DNA in our highly selective mutational systems. The first attempts, performed by Mrs. Z. Opara-Kubinska, employing D98S or D98/AG as recipient cells, treating them with DNA or nucleoprotein isolated from the D98/AGR mutant, and plating on AGR-containing selective medium, were unsuccessful. Similarly unproductive were attempts to transform D98/AG cells to high AH resistance, employing DNA isolated from D98/AH cells. It was then realized that there might be an inherent difficulty in transforming the cell toward loss of enzyme function. Until the IMP pyrophosphorylase was completely diluted out by several rounds of cell replication subsequent to the genetically determined loss of this enzyme function, the addition of AG, AGR, or AH would still result in "lethal synthesis" of the corresponding nucleotides in the "phenotypically unexpressed" cell.

We therefore turned to the reciprocal selective system, employing the strain D98/AH–2, a single-cell isolate of the D98/AH line which produced hypoxanthine-utilizing clones only at frequencies too low for detection by the usual plating methods. Transformants with regained IMP pyrophosphorylase activity, produced by treatment with DNA isolated from D98S or D98/AG cells, would be able to grow and produce colonies on HAT medium. Another favorable feature of this system is the long-term survival of the nonmultiplying D98/AH cells in the HAT medium, which in fact permits them enough time for phenotypic expression, *i.e.*, elaboration of sufficient IMP pyrophosphorylase for colony formation.

Several preliminary experiments were performed, with the D98/AH-2 line as recipient and D98S or D98/AG cells as DNA donors. The DNA was extracted and purified by the method described by Djordjevic and Szybalski (*6*). Freshly trypsinized D98/AH-2 cells, suspended in balanced salt solution containing 4 percent glycerol (to preserve cell viability during treatment) and graded concentrations of spermine (to enhance uptake of DNA), were treated for 30 minutes at 37° C with various concentrations of donor DNA. The cells were then plated in the selective HAT medium and incubated. Control cells were treated with DNA isolated either from T6 bacteriophage or from the recipient D98/AH-2 cells. Colonies appeared only on those plates seeded with cells which had been treated with DNA from D98S or D98/AG cells in the presence of 50 μg spermine per ml. The frequency of these "transformants" increased with the concentration of donor DNA over the range tested (0.5 to 32 μg per ml), approaching 10^{-5} at the highest DNA level. Exposure of donor DNA to DNase completely abolished its activity. The "transformants" were isolated and found to be stable AH-sensitive, hypoxanthine-utilizing clones, which could be distinguished from the spontaneously arising D98/AH-R "revertant" cells on the basis of their survival patterns on various guanine-hypoxanthine analogs. It may be concluded that the phenomenon observed has many features in common with, or is indeed identical to, the DNA-mediated transformation observed in bacteria.

OTHER APPLICATIONS AND CONCLUSIONS

The observations presented here have several theoretical and practical applications, a few of which will be outlined.

(*a*) The mechanisms of resistance development, its frequency and type, could be used as a guide for rational antineoplastic therapy. The selection of therapeutic measures would depend upon whether the clinically observed resistance to purine analogs was of the type of the D98S to D98/AG, or the D98/AG to D98/AH mutation. In the latter case one might hope that treatment with hypoxanthine, amethopterin, and thymidine would lead to elimination of the resistant neoplastic cells in analogy with the behavior of D98/AH in HAT medium (*10*).

(*b*) Use of DNA-mediated genetic transformation might permit the exploration of the fine genetic structure of human genes, especially since the selective systems devised permit the ready isolation of many independent mutants, which are characterized by either loss or gain of IMP pyrophosphorylase activity.

This discussion would not be complete without reference to parallel studies carried out in other laboratories on mutation in mammalian cells affecting resistance to purine analogs (*14–20*) and to attempts to determine mutation rates in mammalian cells grown *in vitro* (*21*). Hopefully, quantitative genetic studies on mammalian cell lines will be further expanded in scope and will supply us with knowledge which at present must be indirectly implied from genetic studies with bacteria and other unicellular microorganisms.

SUMMARY

A prerequisite for quantitative genetic studies is the development of suitable selective markers. This was accomplished by deriving several sequential one-step mutants from the single-cell-isolated human line, D98S. The mutant D98/AG is characterized by a 100-fold increase in resistance to 8-azaguanine (AG), while its derivatives D98/AGR and D98/AH exhibit respective 60- and 7,000-fold increases in resistance to 8-azaguanosine (AGR) and 8-azahypoxanthine (AH). Biochemical studies indicate that the activity of the inosinic acid (IMP) pyrophosphorylase, which converts the corresponding purines and their analogs into "active" nucleotides, is similar in D98S and D98/AG cell-free preparations, although intact D98/AG cells convert AG less efficiently into AG-nucleotide than do the parental D98S cells. D98/AGR cells have lost their IMP pyrophosphorylase activity partially and D98/AH almost completely. The line D98/AH throws off "revertants," which have partially regained IMP pyrophosphorylase activity. Selective systems for isolation and purification of the mutant lines are described, together with methods for determination of mutation rates. None of the putative mutagens tested has shown any high mutagenic activity in the present mutational systems. An attempt to demonstrate DNA-mediated genetic transformation in these cells was successful. IMP pyrophosphorylase-deficient D98/AH cells can be transformed to hypoxanthine-utilizing, AH-sensitive cells at a frequency approaching 10^{-5}, by treatment with DNA extracted from AH-sensitive D98/AG (or D98S) cells in the presence of spermine. Other genetic applications of the mutant lines and mutational systems are briefly outlined.

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DISCUSSION

Studies on virus-cell interaction of polyoma-virus-mediated neoplastic conversion *in vitro*. KLAUS BAYREUTHER, *Division of Biology, California Institute of Technology, Pasadena, California*

The infection with polyoma virus of monolayer cultures of either mouse or hamster embryo cells gives rise to a neoplastic conversion of the culture (*1*). The converted mouse and hamster cultures continue to release polyoma virus indefinitely, and the virus released is invariably a mutant of small-plaque type (*2*).

The continued virus release in mouse or hamster cultures is due to a "virus-carrier state." All its characteristics can be reproduced by infecting nonvirus-releasing clonal sublines with polyoma virus.

Since polyoma-converted cells do not require a multiplying, plaque-forming virus to maintain their neoplastic properties, the presence of the virus in a deficient state in the cells was tested for the presence of: 1) a converting, but noncytocidal virus, 2) an incomplete virus detectable by fluorescent antibody or by its serum-blocking power, 3) an infectious viral nucleic acid, and 4) a provirus susceptible to the inducing conditions effective in lysogenic bacteria. The attempts to demonstrate the virus in any of these states failed.

It is therefore concluded that the cells do not contain the viral genome (*3*).

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Identification and Characterization of Cells by Immunologic Analysis, With Special Reference to Mixed Agglutination [1]

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TO CHARACTERIZE cells growing in culture, there are many ways in which immunologic reactions may be used and further applied as tests for identification. The choice of methods depends to a large extent on whether the histocompatibility antigens of the nucleus, the antigens of the cytoplasm, or the fixed antigens on the cell membrane are selected for characterization.

At present, the histocompatibility antigens have to be studied by transplantation techniques or possibly by "delayed-type" skin reactions. Defendi *et al.* (*1*) established the murine nature of various continuous lines of cells by demonstrating the specific "immune" homograft rejection reaction or "second-set phenomenon."

The complement-fixation test and various modifications of the precipitation reaction, such as double-diffusion agar-gel tests and immunoelectrophoresis, are the obvious methods for revealing the antigenic components of the cytoplasm. These procedures require extracts of the cells and an ample cell volume for study. However, the gel-precipitation techniques offer an ideal method for enumerating and analyzing the various antigenic systems and establishing the identity of these with known antigens (figs. 1 and 2). Coons' method, with fluorescein-labeled antibody, promises to be of great value for demonstrating antigens of both cytoplasm and cell membrane. The preliminary report of Stulberg *et al.* (*2*) illustrates the application of this method in the identification of the species antigens of cultured cells. This method is advantageous because few cells are required for the examination and contaminating minor cell populations can be revealed.

In addition to the fluorescent-antibody technique, the antigens on the cell membrane may be studied by immune cytolysis in the presence of complement (*3*), as well as by special modifications of the agglutination reaction. The empirical finding that the membranes of tissue cells possess antigens common to the red cells of the same species suggested another protocol of investigation to Brand and Syverton (*4*), the latter whose name we are honoring in this symposium. These workers identified the species of cultured cells by injecting the cells into guinea pigs and checking the species-specificity of the hemagglutinins produced.

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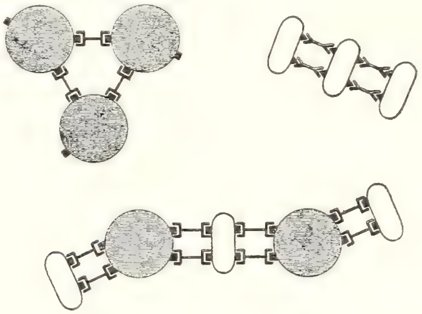
[1] Presented at the Syverton Memorial Symposium on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

The mixed agglutination method described in this paper also depends on the fact that the membrane of tissue cells and red cells of the same species possess what may be regarded as "species-characterizing" antigens in common.

MIXED AGGLUTINATION

Mixed agglutination is the formation of specifically mixed agglutinates of two cell types. In the absence of secondary phenomena, such as those brought about by the involvement of complement and if antibodies of multiple specificity are excluded, mixed agglutination occurs only if the two different cell types possess an antigen or antigens in common. This is understandable on the "Lattice theory" of agglutination, which postulates that an agglutinating antibody is at least bivalent and produces agglutination by specific links bridging one cell to the next (*see* text-fig. 1).



TEXT-FIGURE 1.—No mixed agglutination of different cell types occurs unless the different cell types possess a common antigen.

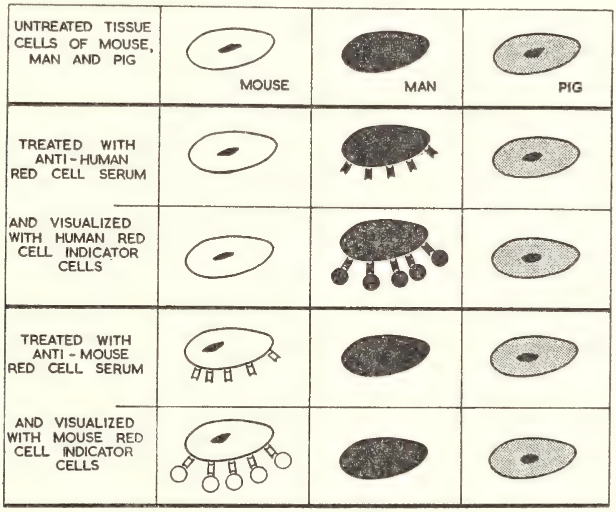
The diagnostic reaction based on this principle of mixed agglutination is essentially a test for antigens on cell membranes and is needed for testing cells such as tissue cells in which, either because of their shape, their tendency to clump spontaneously, or even their scarcity, the ordinary agglutination reaction is excluded. What is necessary for the mixed agglutination reaction is a suitable indicator cell (not necessarily a red cell!) carrying the same antigen or antigens for which the tissue cells are being tested.

DEMONSTRATION OF SPECIES-CHARACTERIZING ANTIGENS OF CULTURED CELLS BY MIXED AGGLUTINATION

As has been mentioned previously, the empirical observation that the membrane of tissue cells and red cells of the same species possess common antigens allows the application of the mixed agglutination reaction as a means of identifying the species of origin of cultured cells.

In our recent work on mixed agglutination (*5*), we have made use of red cell antiserums though theoretically it is possible also to use antiserums made against the cultured tissue cells.

Text-figure 2 illustrates diagrammatically the rationale of the method. The procedure for performing the tests, which has been described in detail (*5*), consists essentially of incubating together the suspension of cultured cells and red cell antiserum, washing away uncombined serum, and lightly centrifuging together the suspension of washed serum-treated cultured cells and red cell suspension of the indicator system. The deposited cells, in a drop of fluid, are transferred to a glass microscope slide and with a coverslip overlaid and sealed on with wax, are examined under a phase-contrast microscope for mixed agglutination. The appearance of a negative and positive test is shown in figures 3 and 4.


UNTREATED TISSUE CELLS OF MOUSE, MAN AND PIG
MOUSE
MAN
PIG
TREATED WITH ANTI-HUMAN RED CELL SERUM
AND VISUALIZED WITH HUMAN RED CELL INDICATOR CELLS
TREATED WITH ANTI-MOUSE RED CELL SERUM
AND VISUALIZED WITH MOUSE RED CELL INDICATOR CELLS

TEXT-FIGURE 2.—Rationale underlying the application of the mixed agglutination reaction for identifying the species-nature of cells in culture. Tissue cells and red blood cells possess species-characterizing antigens in common.

As might be expected, the antiserums have to be absorbed (*5*) to render them specific, and with some systems it has been advantageous to treat the red cell indicator system with papain to increase its agglutinability.

Table 1 shows the specific reaction of these antiserums in mixed agglutination tests on cultured cells and the results of tests on strain L and ERK cells (*6*). Strain L cells react as mouse cells, but there seems little doubt that the ERK cells are human despite the original isolation from rabbit kidney. Table 2 records the results of mixed agglutination tests (*7*) on three other continuous lines of cells, GLRK/7 and GLRK/13 of rabbit origin and GLPK/1 of pig origin. The rabbit nature of the lines derived from the rabbit was confirmed, but the line originating from the pig reacted as human cells. Checks on the species nature of the cell lines KB, HEP, HLM, amnion 9—an adult human fibroblast line of Paul's—and

TABLE 1.—Diagnostic mixed agglutination tests showing specificity of reactions on control panel of cells*

Red cell antiserum and corresponding red cell indicator cell	Cells growing in culture									
	Control panel								Under test	
	Human HeLa	Human KB	Monkey	Rabbit	Pig	Bovine	Rat	Mouse	Mouse L	ERK
Rabbit anti-human	**160**	**160**	20	0	0	0		0	0	160
Guinea pig anti-rabbit	0	0	0	**80**	0	0	0	0	0	0
Rabbit anti-pig	0			0	**20**	0	0	0	0	0
Rabbit anti-bovine	0	0	0	0	0	**640**	0	0	0	0
Rabbit anti-mouse	0	0	0	0	0	0	80	**320**	320	0

*Examination of L and ERK cells for their species identity. Figures indicate titer of serum giving mixed agglutination. Modified after (6).

TABLE 2.—Diagnostic mixed agglutination tests to check the species identity of GLPK/1, GLRK/7, and GLRK/13 lines of cells*

Red cell antiserum and corresponding red cell indicator cell	Cells growing in culture							
	Under test	Control panel					Under test	
	GLPK/1	Human HeLa	Pig	Bovine	Mouse	Rabbit	GLRK/7	GLRK/13
Rabbit anti-human	>40	**>40**	0	0	0	0	0	0
Rabbit anti-pig	0	0	**20**	0	0	0	0	0
Rabbit anti-bovine	0	0	0	**640**	0	0	0	0
Rabbit anti-mouse	0	0	0	0	**320**	0	0	0
Guinea pig anti-rabbit	0	0	0	0	0	**80**	40	40

*Figures indicate titer of serum giving mixed agglutination. Modified after (7).

MCN—studied by Dr. Moor-Jankowski (*8*) in our laboratory—confirm the human origin of them all except MCN which reacts as a mouse cell.

DIFFERENTIATION OF CLOSELY RELATED SPECIES

If a reagent is required to differentiate between cells of closely related species such as the mouse and rat, it is probably better not to attempt to make a rabbit antiserum specific by absorption with the heterologous tissue of the closely related species, but to produce the red cell antiserum in the closely related species in question. For instance, to have specific reagents differentiating mouse and rat tissue cells, the serum specific for mouse tissue cells should be made in the rat and the red cell antiserum specific for rat tissue cells should be made in the mouse. The reactions of serums (*5*) illustrating this point are shown in table 3. Dr. Franks (*9*) in our laboratory is making use of this principle to differentiate human and monkey cells.

TABLE 3.—Mixed agglutination tests differentiating rat and mouse fibroblasts*

Red cell antiserum	Indicator red cells	Cells in culture				
		Human HeLa	Ox kidney epithelium	Pig kidney epithelium	Rat fibroblasts	Mouse fibroblasts
Rat anti-mouse	Mouse	0	0	0	0	16
Mouse anti-rat	Rat	0	0	0	32	0

*Serum diagnostic for mouse tissue cells was made in rats; serum for rat tissue cells, in mice. Figures indicate titer of serum giving mixed agglutination (*5*).

Heterophil antiserums, such as anti-Forssman may also be of value in differentiating between closely related species; *e.g.*, chicken and duck fibroblasts and mouse and rat cultured cells should be distinguished by anti-Forssman serums.

HETEROPHIL ANTIGENS AS MARKERS IN IDENTIFICATION

Heterophil or heterogenetic antigens are antigenically related substances in the cells of animals (microorganisms and plants) which are widely separated in the zoological system. The group of Forssman antigens forms the best known example: Forssman antigens may be demonstrated on cells by mixed agglutination as they occur as part of the membrane of tissue cells, and red cells possessing the antigen are available for the indicator system.

An extensive study using anti-Forssman serums in mixed agglutination tests on cultured cells (*10*) has been undertaken: Cells from man, rhesus monkey, rabbit, pig, rat (with a certain *proviso*), and bovine cells react negatively while cells from the guinea pig, horse, fowl, cat, and mouse react positively.

Unlike the species-characterizing antigens discussed in the previous section, the Forssman antigen may be acquired by cells (innately negative-reacting) if they are grown in medium containing serum from Forssman-positive animals or possibly some other source of this widely distributed antigen.

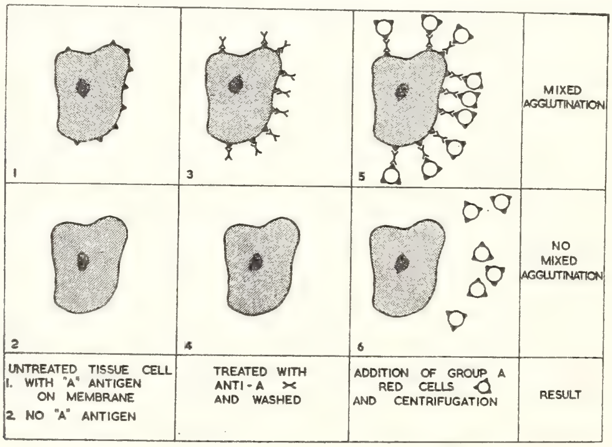
It is interesting that the L cells we tested possessed no Forssman antigen, yet mouse cells do. An investigation is now in progress to see how long cells, isolated from a Forssman positive animal, maintain the antigen in a medium devoid of the antigen.

From the point of view of the present symposium, it should be remembered that there are other heterophil antigen-antibody systems, and these should be investigated in the hope that they too will afford further markers for cell characterization and identification.

ISO-ANTIGENS AS MARKERS IN IDENTIFICATION

Iso-antigens are genetically determined antigens varying within the species. Actually, it was to examine platelets and epidermal cells for the ABO blood group iso-antigens that the mixed agglutination reaction was originally introduced by us in 1955 and 1956 (*11, 12*).

Text-figure 3 reminds us again of the procedure adopted for the examination of tissue cells for the blood group A iso-antigen. Examination of HeLa cells for blood group iso-antigens by mixed agglutination (*13*) revealed the H, MN-type, and Tja antigens. Other established human lines are now being tested by Dr. Moor-Jankowski in our laboratory for these and other iso-antigens, and differences in the antigenic composition are being revealed. If this iso-antigenic structure is subsequently shown


1
3
5
MIXED AGGLUTINATION
2
4
6
NO MIXED AGGLUTINATION
UNTREATED TISSUE CELL
1. WITH "A" ANTIGEN ON MEMBRANE
2. NO "A" ANTIGEN
TREATED WITH ANTI-A ✂ AND WASHED
ADDITION OF GROUP A RED CELLS AND CENTRIFUGATION
RESULT

Text-figure 3.—Rationale underlying the application of the mixed agglutination reaction for revealing the presence of the A iso-antigen on tissue cells.

to be basic to, and constant in, the different cell strains, then such markers will be available to differentiate cell lines within a species.

For this reason it is perhaps a little disappointing that, as yet, so few of the human red blood cell iso-antigens have been demonstrated on cells other than the erythrocyte. The position in species other than man must also be investigated. The recent advances in animal blood grouping should mean that specific reagents are now available with which to examine established lines of animal cells in the hope of characterizing the lines in antigenic terms.

Finally, an antigen such as the human A or B blood group antigen may act as an iso-antigen in one species, *i.e.*, the B antigen in man, and as a species or heterophil antigen in another species, *i.e.*, the B antigen in the rabbit, mouse, and pig.

SUMMARY AND CONCLUSIONS

Brief mention of some immunologic methods available to identify and characterize cells in culture has been made.

The potentialities of the mixed agglutination reaction have been discussed in more detail.

As with nearly all immunologic methods, each of the methods I have mentioned has, besides its special advantages, its own limitations, and special precautions in interpreting the results may be required.

The full value of these methods will only be realized when the numerous antigens of the different cells have been fully mapped and analyzed. Then not only will the methods be useful for purposes of identification, but they will also provide a possibly unrivaled means of studying the changes in cells associated with *in vitro* transformation or *in vivo* malignant change.

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Plate 5

Figures 1 and 2.—Antigens in extracts of cultured cells demonstrated and identified by double-diffusion precipitation tests in agar-gel; an experiment of Dr. Kelus showing differentiation between mouse cultured cells L and cultured cells of human origin: HeLa, AM 9, and the controversial cell ERK 1. Lines of precipitation also show common antigens shared by cultured cells and tissues in organs of the animal species in question.

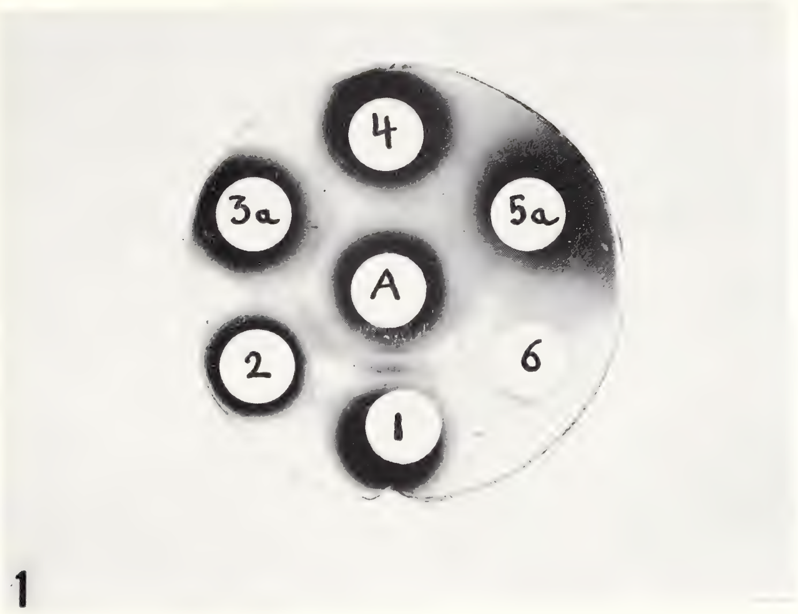
KEY

In Central Well A: Rabbit antiserum produced against ERK 1 cells cultured in medium of Parker 199 and rabbit serum.

In Central Well B: Rabbit antiserum produced against L cells grown by Dr. Merchant in protein-free medium.

Contents of Peripheral Wells

1	Extract of ERK 1 cells
2	Extract of AM 9 cells (human amnion)
3a	Extract of human liver
3b	Extract of mouse liver
4	Extract of L cells
5a	Extract of human spleen
5b	Extract of mouse spleen
6	Extract of HeLa cells



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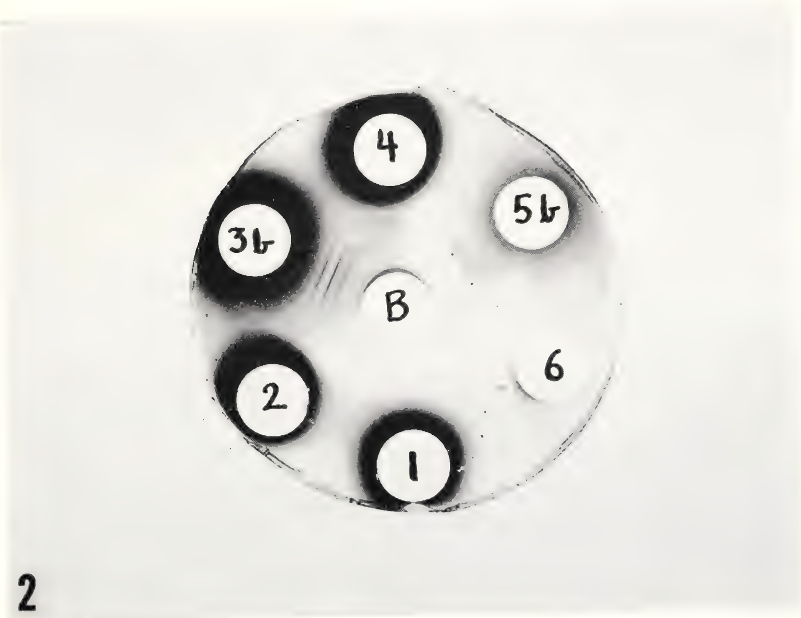


2

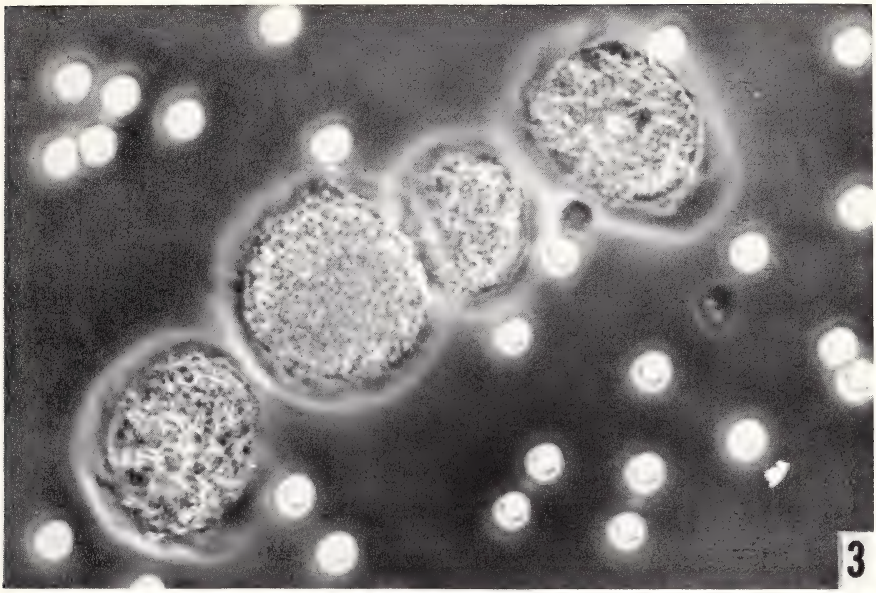
COOMBS

Plate 6

The appearance of a mixed agglutination reaction on cultured cells to demonstrate their species of origin. Phase-contrast microscopy, × 10 eyepiece × 40 objective.

Figure 3.—Negative reaction.

Figure 4.—Positive reaction.



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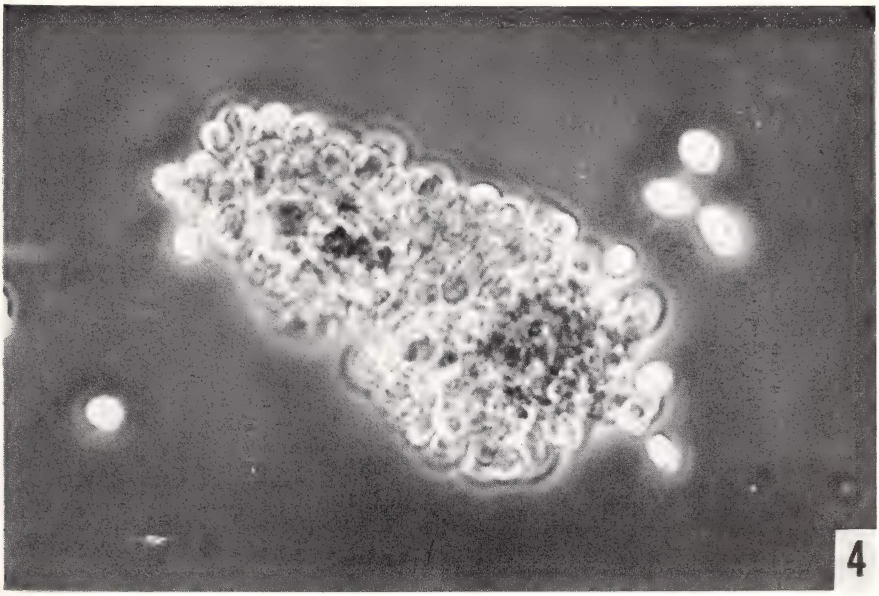

4

DISCUSSION

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I am very much impressed by Dr. Coombs' presentation and his very interesting and elegant method.

I think I should add a few remarks on the investigations, similar to those of Dr. Coombs, which Dr. Syverton and myself carried out 2 years ago. By using primary cell cultures and established nontransformed cell strains for species-determination, our species-diagnosis was always in agreement with the claimed species-origin. This was not so, however, with most of the 19 "transformed" strains tested. With these investigations we hoped—and this was the main purpose of our work—to help clarify the confusion in connection with the so-called cell-transformation phenomenon. Our data obviously supported the opinion of those who believed that this drastic form of cell transformation with changing species characteristics might be due to contamination with subsequent overgrowth of slowly propagating cell cultures by cells of foreign, well-established, and faster-multiplying strains. In this respect it is of interest to note that cell transformation in those strains that we have investigated always resulted in appearance of either mouse or human species characteristics. This suggested the possibility of contamination with established cell strains most commonly used in laboratories such as mouse strain L or possibly human HeLa.

The test used in our studies was a simple hemagglutination test different from Dr. Coombs' test insofar as the *cultivated cells* were injected into groups of guinea pigs to evoke species-specific antibodies which agglutinated erythrocytes of the corresponding species. The specificity was confirmed by cross-absorption and other experiments to exclude the action of blood group, heterophil, or heterogenetically induced agglutinins. Cross-absorption tests were carried out routinely whenever we had to differentiate human and monkey cells.

There are, however, some disadvantages in our test as a *simple routine* procedure: 1) Cultivated cells of certain species are poor inducers of species-specific hemagglutinins in guinea pigs. Therefore, it may become necessary to booster the animals or even to try another animal species for immunization. 2) *Groups* of animals rather than single animals must be used for immunization in order to avoid false diagnosis from occasional nonspecific antibody response in individual animals. 3) Relatively large numbers of cells must be used, especially if cross-absorption tests become necessary, as for the differentiation of human and monkey cells. And 4), it takes at least 3 to 4 weeks until results can be obtained.

These disadvantages apparently do not play a role either in Dr. Coombs' test or in Dr. Stulberg's application of the fluorescent antibody technique. Therefore, I think both methods are very useful for routine purposes. However, it seems to me that Dr. Coombs' method also introduces a certain disadvantage in that it might be difficult to analyze mixed or contaminated cell cultures. During our investigations, we have detected such mixed cultures on several occasions. The sensitivity of our method in this respect was determined on an artificial mixture of 1 rabbit cell cottontail rabbit epithelium (CRE) per 1,000 human cells esophageal epithelium (EE), worked up in our laboratory as an unknown. The data obtained clearly demonstrated the presence of both species. Thus the sensitivity of our method certainly was of value for the interpretation of results obtained on transformed cell strains.

There are some other points of interest to me that I would like to bring up. I wonder whether the antibodies measured in Dr. Coombs' test and in our test are identical. The antibodies we are measuring are obviously directed against species-specific proteins. In Dr. Coombs' test carbohydrates might be involved, since agglutination titers apparently can be increased by papainization of erythrocytes, as indicated in one of Dr. Coombs' recent publications (Nature 189: 503–504, 1961). In our test species-specific hemagglutination titers are considerably reduced by trypsinization or papainization of erythrocytes.

Furthermore, I wonder whether the antigens Dr. Coombs is dealing with are only located on the cell membrane or in other parts of the cell, too. I ask this question since we have found in species-specific hemagglutination inhibition tests with cellular fractions that the species-specific *proteins* are distributed throughout the cell, even, though in very small amounts in the purified nuclear fraction. The power of the different cellular fractions to inhibit species-specific hemagglutination correlated quantitatively with the protein content of each of the fractions.

Finally, I would like to remark on the necessity for determination of cell strains within species. If cell contaminations occur frequently *between* species, one must expect it also may occur *within* species. So, we must consider the possibility that some cell lines of the same species carrying various designations actually might have been derived from previously established cell strains by way of exogenous cell contamination. Therefore, I think we are in need for methods to classify cell strains within species. An immunological test of this type would, of course, require extreme sensitivity in order to detect very small antigenic differences. This, on the other hand, increases the danger of nonspecific reactions, as the entire complexity of cell antigenicity comes into play as well as antigenic additives from the media or even from latent microbial contaminations of cell strains. The application of Dr. Coombs' "mixed agglutination test" for demonstration of antigenic markers within species appears to be a promising approach.

Methodology of Chromosomal Analysis in Man[1]

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THE REMARKABLE advances made during the last few years in mammalian and particularly human cytogenetics are the natural outcome of the introduction of new technical principles. Of these the use of tissue culture is one. Its value, of course, is that it provides an excellent source of cells in mitosis. The cells, moreover, are usually larger and have more abundant cytoplasm than most of the naturally proliferating cells within the body. This is an important advantage for the squashing and air-drying procedures to be mentioned later since the chromosomes are so readily dispersed.

The counting of chromosomes and the determination of their morphological features are best done at metaphase. They are then shorter and more distinct than at any other stage of mitosis. But in the natural mitosis they lie closely packed, with their centromeres in the equator of the spindle and their arms lying irregularly above and below. Their separate resolution is therefore a matter of difficulty and, even with the best preparations made by the classical procedure of embedding tissues in paraffin wax and sectioning, observation was tedious and uncertain. The microscopist had to build up his picture by integrating from a series of optical sections. Drawings were at a premium and photomicrographs were almost useless. Most of the technical innovations have therefore been concerned with dispersal of the chromosomes within the cell so that they could be seen clearly and counts and observations of morphology made faster and more accurately.

The technical devices contributing to better chromosome dispersal include the adaptation of the squash method of Heitz (*1*) for mammalian material (*2–4*); exposure to hypotonic fluids to swell the cells and prevent spindle formation (*5, 6*); the adoption from plant cytogenetics of the spindle-inhibitor, colchicine, and later its analogue desacetymethylcolchicine (Colcemid, CIBA), to accumulate mitosis at metaphase and to shorten and disperse the chromosomes (*7, 8*); and the air-drying of cells from an alcoholic fixative as an alternative to squashing (*9, 10*). By the use of some combination of these devices the chromosomes are spread widely within the cell, ideally in a single plane, without overlaps. The contemporary cytogeneticist therefore works essentially in two dimensions rather than three and can often provide excellent photographs as objective evidence of his findings.

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[1] Presented at the Syverton Memorial Symposium on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

[2] The author wishes to thank Mr. G. Breckon for the preparation of the illustrations.

Not only is dispersion of the chromosomes greatly improved in the best contemporary preparations, it is probable that the sharpness of fixation and the clarity of staining are improved also. The explanation may lie in rapidity of penetration. To employ the squash or air-drying methods to best advantage cells are often processed as suspensions, *e.g.*, ascites tumors (*11*, *12*), bone marrow (*13*), and tissue cultures (*9*). The fixative and other fluids would inevitably penetrate more rapidly than when even small pieces of tissue are processed in bulk. The same considerations should also hold when coverslips bearing a monolayer of adherent cells are processed.

It is possible to obtain very satisfactory squash preparations from the bone marrow of mammals that have previously been injected with colchicine (*13*). Figure 1 illustrates a tetraploid cell from the bone marrow of a guinea pig, in a preparation made by this method in 1956. Essentially the same procedure has been used for human patients (*14*). Few clinicians, however, are prepared to inject colchicine or Colcemid into their patients to improve the prospects of technical success. It therefore appeared to be necessary to have dividing cells that could be exposed to the action of these agents outside the body. Recently, however, Sandberg *et al.* (*15*) succeeded in obtaining preparations from marrow biopsy specimens taken from leukemic patients who were *not* injected with a spindle-inhibiting drug beforehand. The cells were exposed to a hypotonic solution and subsequently fixed and squashed in acetic orcein. Whether or not this simple procedure can become a fully efficient general method remains to be seen.

The main development of techniques for the study of human somatic chromosomes has, in fact, turned on the provision of dividing cells *in vitro*. There are three general methods. They depend upon the short-term incubation of bone marrow cells, true tissue culture, and the culture of white cells from the peripheral blood, respectively.

METHODS

Bone marrow.—This method (*16*, *17*) was originally developed for studies of chromosomes in leukemic cells. The cells from a marrow biopsy specimen are first dispersed in glucose saline. The suspension so obtained is next mixed with approximately 4 times its volume of human AB serum and incubated *in vitro* at 37° C just long enough to ensure that an adequate number of cells have entered mitosis. This takes about 7 hours. The cultures are then injected with colchicine (or Colcemid) and finally processed as for mammalian bone marrow. The short-term culture method itself was devised for metabolic studies (*18*, *19*).

Tissue culture.—Although Kemp (*20*) used tissue cultures over 30 years ago in an attempt to verify the normal number of human somatic chromosomes, the potentialities of this approach remained unappreciated until Hsu (*21*) demonstrated the advantages to be gained by subjecting cells in culture to hypotonic treatment before fixation.

Tjio and Puck (*10*) were the first to describe a method of general applicability. Minute skin biopsy specimens were subjected to trypsin digestion and the suspension of cells obtained was used to seed primary cultures. Subsequently Puck, Robinson, and Tjio (*22*) reported that the scabs produced by lightly scarifying the skin form an excellent source of cells that will proliferate in culture. Harnden (*23*) also succeeded in establishing cultures from small skin biopsy specimens. These were cut into pieces about 1 mm square and attached to plasma clots. It is worth noting that a piece of skin as small as 1 × 3 mm is ample to establish two or three primary cultures. The cut should just enter the dermis and no more. Edwards (*24*) described a simple method for taking specimens. The presumption is that the cells which grow out are fibroblasts of the dermis, though this is not proved.

Cultures can also be established from explants of deeper-seated tissues or organs and the opportunity to obtain specimens may arise from time to time at operation or autopsy. Lejeune (*25*) has used fragments of fascia lata taken under total anesthesia. Fraccaro, Kaijser, and Lindsten (*26*) established successful long-term cultures from specimens of bone marrow.

Once healthy, dividing cells have been obtained in abundance, cytological processing can follow any of a great variety of procedures (*9, 10, 23, 25, 27*). Although it has been possible to obtain preparations from primary cultures (*25*), it has been more usual to wait until the cells are growing vigorously in subculture. As already indicated, the cells can be harvested and processed as suspensions, or coverslips can be placed in the culture vessels overnight and the adherent cells pretreated, fixed, and stained the next day.

Blood culture.—The culture of white cells from venous blood is in many ways the most efficient of the three methods and is now being used widely. It was first employed to provide chromosome preparations by Nowell, Hungerford *et al.* (*28, 29*) who made use of an adaptation of the gradient culture method of Osgood (*30, 31*). A full account of the method incorporating improvements has been given by Moorhead *et al.* (*32*).

From 10 to 15 ml of venous blood is sufficient to set up two or three culture bottles. The red cells are precipitated. The phytohemagglutinin used for this purpose was subsequently discovered to be a powerful stimulant of mitosis (*33*) and is now recognized to be an essential feature of the technique. The plasma containing the white cells is mixed with tissue-culture medium 199 in the proportion of 1 part of plasma to about 5 parts of medium. Aliquots are incubated in culture bottles at 37° C for 3 days, or 2 days if the blood contains leukemic cells.

Successful cultures at this time contain a high proportion of large mononuclear cells, many of which are in mitosis. There is a decline in the concentration of cells in the medium during the period of culture, the loss of polymorphonuclear cells being disproportionately high. At first it was considered likely that the large mononuclear cells are derived from monocytes and large lymphocytes (*33*), but present opinion seems to favor the view that they originate from small lymphocytes, though no supporting evidence has yet appeared in print. A typical mitotic cell in an air-dried preparation from a blood culture is illustrated in figure 2.

It may not always be possible to secure sufficient blood for the procedure just outlined, and it is therefore of value that Edwards and Young (*34*) have devised a method whereby cultures can be set up and a few preparations obtained from as little as 1 ml of blood. This method is likely to be particularly useful if the subject for investigation is an infant since the minimum volume of blood required may be obtained from a heel prick.

THE THREE METHODS: PRO AND CON

The bone marrow method has the obvious disadvantage that the biopsy is an unpleasant experience for the patient, and it is the most difficult of the three methods technically. On the other hand, the interval between setting up the cultures and harvest is so short that we can be quite sure that the cells seen in mitosis actually existed within the body.

Cultures established from skin have opposed characteristics: The biopsy procedure is least traumatic to the patient, but the final preparations are made from cells that are the product of many divisions *in vitro*. In some circumstances, cells may be maintained in culture for many transfer generations without detectable alteration of the karyotype (*10*). Nevertheless the possibility of karyotypic changes arising and being perpetuated, perhaps selectively, should always be borne in mind. A case reported by Carr *et al.* (*35*) illustrates the danger. Their first observations were made on cells of a single culture line in which vigorous growth was observed after a period of quiescence. They revealed a highly abnormal karyotype which included two chromosomes in a total of 48 that were morphologically abnormal. However, preparations from further cultures and observations of sex chromatin left little doubt that the patient was a straightforward XXXY case, and that the original observations were made on a clone of karyotypically abnormal cells that had arisen in culture and overgrown all the rest. This provides a warning to set up cultures in parallel wherever possible.

Blood culture is now undoubtedly the method of choice. There is little disturbance to the patient, and the preparations can be obtained within 3 days. It is not yet known how many successive mitoses may occur during the period of culture, but it is highly unlikely to be more than two or three and it may well be that, as with the bone marrow method, the observations are made on cells in their first mitosis after removal from the body. Even should a karyotypic change occur very early, it could hardly lead to the formation of a clone of more than four cells; the recognition of the unaltered karyotype should therefore not be prejudiced.

In view of the occurrence of mosaicism, particularly in patients with abnormal numbers of sex chromosomes, the determination of a karyotype from a single primary tissue specimen cannot be regarded as unequivocally representative of the patient. In all cases, and especially the anomalies of the sex chromosomes, additional determinations are desirable.

The three methods also complement one another for the study of the chromosomes in cases of leukemia. Examination for this purpose by both the bone marrow and blood-culture methods is obviously desirable. Should these methods reveal a small morphological change but no alteration of number (*36*) it would then be essential to test the possibility that the abnormality is a feature of the patient rather than the disease by growing normal cells for examination from a tissue such as skin.

IDENTIFICATION OF INDIVIDUAL CHROMOSOMES

The identification of the individual chromosomes is a topic which appropriately falls within the ambit of methodology. The Denver system of nomenclature (*37*) has been widely used, notwithstanding its acknowledged imperfections. The karyotype of a normal male arranged according to this system is reproduced in figure 3. The primary characteristics used for identifications of individual chromosomes are, of course, relative length and centromere position.

Autosome pairs 1, 2, and 3 [Group A of Patau (*38*)] are clearly quite distinct from one another and from all the remainder. Pairs 4 and 5 (Patau's Group B) are very similar and were not distinguished in the Denver report. It is the medium-sized chromosomes, including autosomes 6 to 12 and the X (Patau's Group C), that have caused the greatest difficulty, and Patau has asserted that they cannot be distinguished by present methods (*38*, *39*).

Pairs 13, 14, and 15 (Patau's Group D) are the three pairs of long acrocentric chromosomes. At the time the Denver report was published it was believed that two pairs bore satellites and that they could be distinguished by size. It is now known that satellites are carried by all three, so that the Denver distinction is invalid.

It is our experience that pairs 16, 17, and 18 (Patau's Group E) are somewhat variable in their appearance, possibly due to natural polymorphism. Pair 16 is one of the most individual types of the whole complement, and can often be recognized at a glance. We find that pairs 17 and 18 are sometimes clearly separable.

The two small metacentric pairs, numbers 19 and 20 (Patau's Group F) are difficult to separate, and so are the two small acrocentric pairs (Patau's Group G). It was at first believed that the latter could be distinguished by presence or absence of satellites, but it is now known that both bear satellites. All ten of the acrocentric chromosomes may therefore be satellited. It is necessary, however, to point out that the satellites are not always seen: I have not recorded more than 6 in any one cell. The number of visible satellites varies considerably between one cell and another of the same individual. The Y chromosome is also a member of Group G. It varies in length between different males, sometimes being appreciably longer than either of pairs 21 and 22. It has a more nearly terminal centromere, its chromatids tend to lie closer together than the chromatids of the other chromosomes, and it has never been seen to bear a satellite.

Notwithstanding the great similarity within the seven groups just discussed, my co-worker, Dr. C. M. Clarke, and I believe that all the 24 individual types of chromosome can be validly distinguished on the basis of the three features of relative length, centromere position, and satellite size. This is not the place to give our evidence at length: We hope to do so elsewhere.

Needless to say, additional morphological markers on human somatic chromosomes would be most valuable. One of the chromosomes of the 6–12–X group sometimes bears a secondary construction close to the centromere (*39*), and it has been claimed that in certain circumstances the X chromosome is heteropyknotic at metaphase (*15*), but these features are still too ill defined to be of general value in identification.

There remains the possibilities of inducing specific morphological changes by treatment. These include the revelation of heteropyknotic regions by cold treatment, as in plants (*40*), and the positive identification of the nucleolar organizers, perhaps by the use of cobalt salts to induce nucleolar persistence into metaphase as Heath has found in cultures of chick fibroblasts (*41*). Work on these lines would be valuable.

FINAL COMMENTS

Excluding the final microscopy, the three methods we have considered are naturally divisible into two phases: 1) the provision of cells in mitosis and 2) the series of manipulations that intervene between living cells and final preparations: in brief, into culture and microtechnique. Hitherto these have been different provinces requiring different backgrounds, experience, and skills so that much of the most fruitful work has been done in collaboration between investigators with different trainings. However, since the two phases can each be regarded as practical applications of cell physiology, the future may produce a breed that combines mastery of both. The procedures are still highly empirical, but as knowledge increases it is to be hoped that our understanding and control of what we do when we grow our cells and make our preparations will become more secure.

For the present we are left with no alternative but to recognize the cookbook character of our methods and to proceed accordingly. The different microtechnique components of pretreatment, fixation, dispersion of the chromosomes, and staining can be varied endlessly and a successful combination probably can always be improved. Slavish adherence to routine is therefore to be discouraged.

It should not be expected that even the most careful attention to the details of a published schedule will ensure immediate success. Furthermore it is probably by no means an uncommon experience that a well-established procedure suddenly fails to produce satisfactory results for no obvious reason. The experienced technician may be able to put his finger immediately upon the cause of the trouble: Others may have to test the individual steps systematically. Failures doubtless arise because so many variables are involved that rigorous definition of them all eludes us. The art of making good preparations is still an art.

SUMMARY

An outline is given of the three principal procedures in current use for obtaining preparations to study human somatic chromosomes, namely, short-term incubation of cells from bone marrow, long-term culture from skin or other tissues, and culture of white cells from the peripheral blood. Their advantages and disadvantages are discussed. Present knowledge of the characteristics of individual human chromosomes is summarized.

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Plate 7

Figure 1.—Tetraploid cell of guinea pig, $4n = 128$. Feulgen squash preparation of bone marrow. × 2,300

Figure 2.—Diploid cell of human female, $2n = 46$. Air-dried preparation of cultured peripheral blood. Acetic orcein stain. × 1, 650

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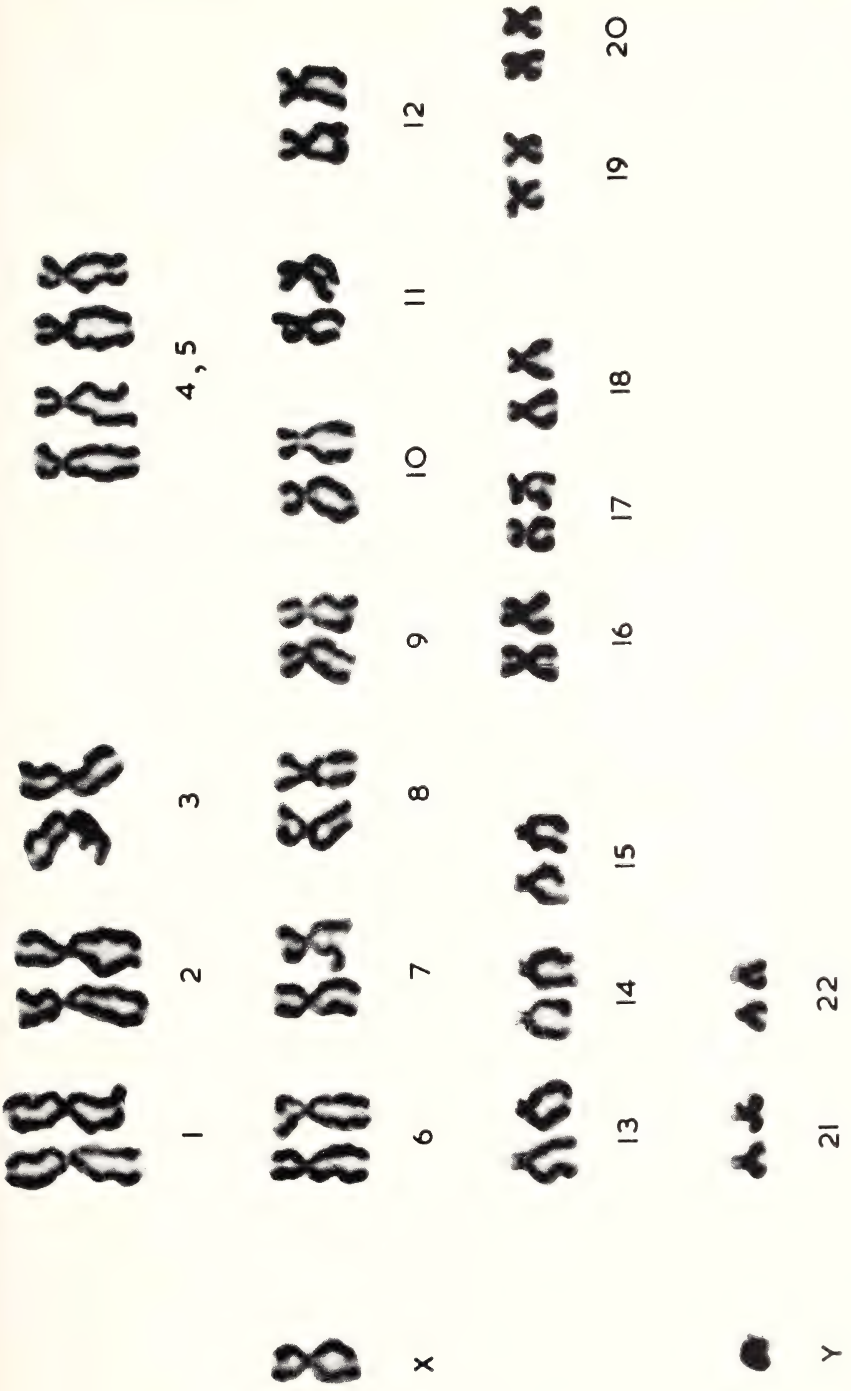
PLATE 8

FIGURE 3.—Karyotype of normal human male. Distinction between pair #4 and #5 is not possible in this cell. Orcein-stained preparation from skin culture. × 3,200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DISCUSSION

P. S. Moorhead, *Wistar Institute, Philadelphia, Pennsylvania*

Dr. Ford has ably considered the major methodologies for chromosomal analysis of human cells. His emphasis upon a flexible or eclectic use of *all* these techniques seems important to me in relation to our future work in finally delineating the human karyotype and in studies of human chromosomal pathology.

Although our advances have been striking over the past 10 years, a brief consideration of the extent of our ignorance may also be of value.

Even the basic human idiogram as originally presented by Tjio and Levan (*1*) and confirmed and extended by many workers remains incomplete. For scientific validation and acceptance the particular cytologist's reputation and experience still remain important considerations. Our position is like that revealed in the well-known definition of a species: "A species in a particular group of organisms is that which a qualified taxonomist with knowledge of that group states so to be." To avoid this kind of "science by authority" we must base new findings and their acceptance on demonstration.

Conservative opinion states that no more than 6 pairs of the human complement are identifiable. Other investigators have claimed identification of practically all 23 pairs.

Small but intriguing differences in length between pairs #4 and #5, #19 and #20, and between certain members of the #7 to 12 group have been noted and accepted by some workers as reliable and valid. Speaking only from my own experience, I feel such differences will prove to be intrinsic and characteristic of the chromosomal pairs cited. However, the clear demonstration of such differences to the skeptic is not usually possible. Chromosomal length variations are surprisingly large. Even when known to be present (as between #1 and #3) differences in length between nonhomologous chromosomes are often obscured by the range of degree of coiling or by artifacts of spreading techniques.

An excellent example of the approximate limit of *demonstrable* differences in length is demonstrated in the Philadelphia chromosome found by Nowell and Hungerford (*2*) to be associated with chronic granulocytic leukemia. The slightly shorter length of this chromosome can be noted by comparison with members of the #21 to 22 group.

Extension of our detailed knowledge of the human chromosomal pairs may *still* depend heavily upon careful, meticulous microscopy. An example of an accessory detail or individual marker for a human chromosome is shown in figure 1. This secondary constriction near the centromere has been observed frequently in our preparations. In Hungerford's choice of nomenclature and my own, this is pair #9 (*3*). Patau (*4*) has described this chromosome and termed it C′. Such accessory markers for the human chromosome pairs are valuable and others may yet be found. A *rarely* observed secondary constriction is shown in figure 2. *Note* chromosome #4 (or #5) and its presumed homologue nearby, each with a constriction just off center in the short arm. We have seen this in only 3 separate instances. Its rarity, of course, forces us to reject it as a valid or reliable feature. To aid us in completing our knowledge of the human karyotype it may prove useful to use such compounds as 5-bromodeoxyuridine which Hsu and Somers (*5*) have shown can induce an exaggeration of secondary constrictions.

More imaginative approaches and methods are needed to exploit chromosomal features yet unknown and to correlate these with cell morphology. In this direction, the hypothesis that the acrocentric association commonly seen at metaphase reflects the fact that the 5 pairs of acrocentrics are involved in nucleolar formation has recently received additional support from Ohno and coworkers (*6–8*).

Apart from these beginnings, we know little concerning the nature of the chromosomes during interphase. Warren Lewis and E. B. Wilson, many years ago, emphasized the chromosomal nature of the karyomeric vesicles which comprise the interphase nucleus. The retention of separate vesicles is barely indicated in a presumed tetraploid nucleus from a human diploid cell strain of Hayflick's (fig. 3). The integrity of the chromosomes through interphase is also reflected in figure 4, showing a linear array of granules from an autoradiograph of a diploid human cell.

It may be hoped that classic plant and Drosophila cytogenetic principles can be extended to include such methods as the induction of differential or regional staining by cold shock or other insults to the cell. This condition is here shown in a human diploid cell but was *not* produced by treatment (figs. 5 and 6), it being only a chance finding. However, its existence teases us and promises even more interesting developments in human cytogenetics.

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Plate 9

Figure 1.—Secondary constriction near base of long arm of chromosome of #7 to 12 group. Regarded as pair #9. Peripheral blood leukocyte from "normal" female, air-dried. × 2,600

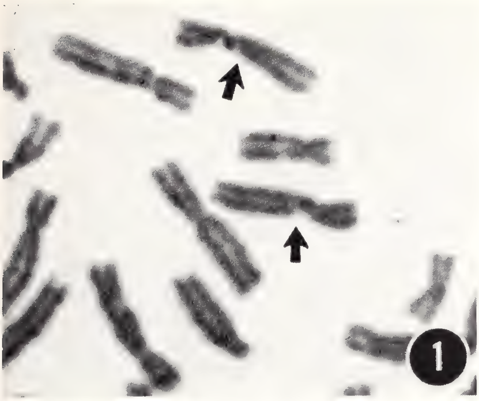
Figure 2.—Rarely observed secondary constriction in short arm of two chromosomes of #4 to 5 group. Same cell as in figure 1. × 2,600

Figure 3.—Presumed tetraploid nucleus from human diploid cell strain. *Note* separation between adjacent karyomeres. × 1,500

Figure 4.—Autoradiograph of nucleus of cell of human diploid strain. × 1,100

Figure 5.—Differential staining along chromosomes of cell of human diploid strain. Fixed directly in aceto-orcein stain and squashed. × 2,300

Figure 6.—Different focal level of cell in figure 5. × 2,300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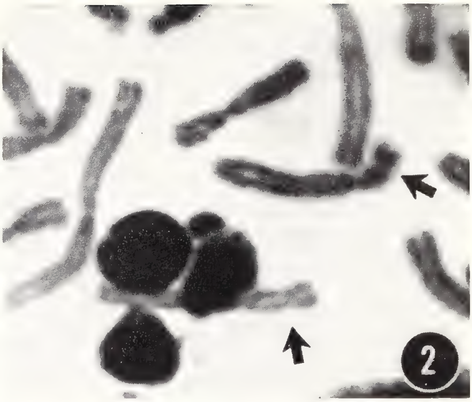
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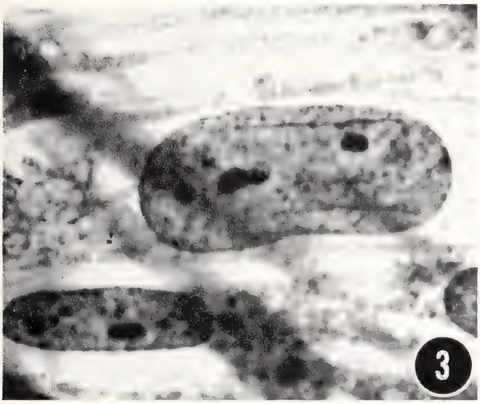
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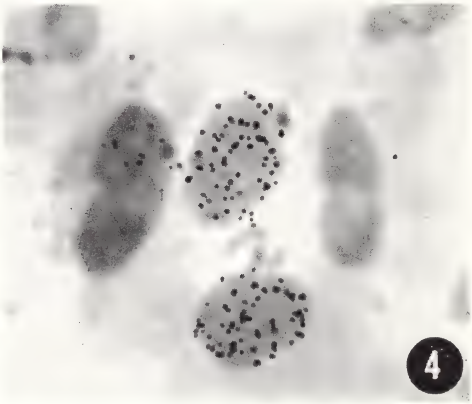
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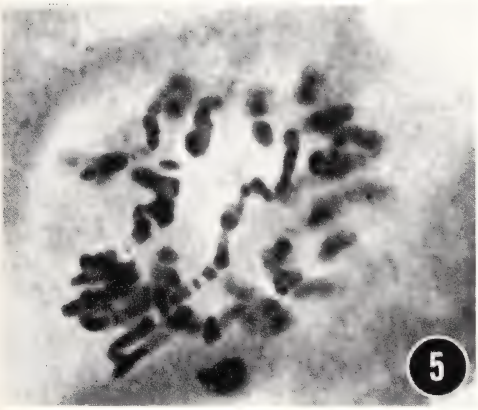
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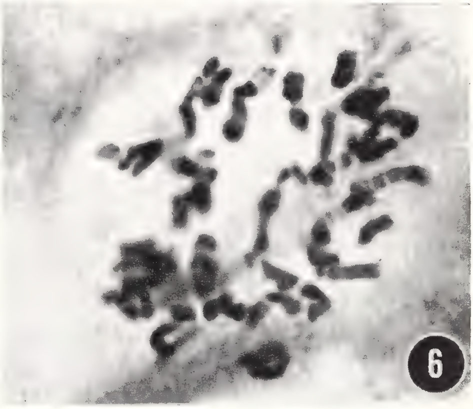
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Cytological and Cytochemical Characterization of Cells Grown *In Vitro* [1, 2]

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ALTHOUGH TISSUE CULTURE has passed through its taxonomic phase, highlighted by the classical descriptions of Lewis and Lewis (*1*), Strangeways (*2, 3*), Ludford (*4, 5*), Richardson (*6*), Hill (*7*), MacDougald (*8*), Bourne (*9*), and Fell and Hughes (*10*), structure is still fundamental to the characterization of cells cultivated *in vitro*. Unfortunately, morphologic criteria have been largely ignored in recent years, except for several studies on the ultrastructure of cells (*11–13*). This is understandable since structural differences tend to become indistinguishable (*14*) when cells are in continuous cultivation for long periods. Consequently, the cell biologist has turned to quantitative methods as more critical and perhaps more revealing indexes of cell metabolism and function. However, technical limitations have required that such studies be performed on cell populations with little regard to the variability between individual cells observed within such populations.

Our interest in the cytological and cytochemical parameters of mammalian cells grown *in vitro* was prompted by the observation that strain L or LLCM$_1$ mouse fibroblast cells grown in suspension could be stimulated to form membranous structures *in vitro* (*15*). While numerous workers reported fiber formation in primary explant cultures of fibroblasts (*16, 17*), there were no such reports concerning monolayers or suspension cultures of established cell strains. Since then, Pumper (*18*) reported similar findings in mouse lung cells and Kuchler, Marlowe, and Merchant (*19*) postulated mechanisms involved. Suspension cultures, maintained in synthetic medium 199 (*20*) supplemented with 1.0 percent Bactopeptone (Difco Laboratories, Detroit, Mich.) and agitated on a rotary shaker, yielded delicate membranes at the fluid-air interface. These membranes contained a number of pleomorphic cells dispersed among large numbers of fibers arranged singly and in bundles (fig. 1). Although the rounded cells in suspension ranged from 12 to 18 μ in diameter, these spindle-shaped cells found in the membranes ranged from 18 to 145 μ in length. With the Masson technique, they varied in tinctorial

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[1] Presented at the Syverton Memorial Symposioum on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

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[3] The authors are deeply indebted to Mrs. Julia Ternak and Miss Denise Strelbitzky for their technical assistance.

properties from smaller cells, exhibiting a lightly stained nucleus and fine eosinophilic granules in the cytoplasm, to large, sometimes multinucleate cells, which displayed a deeply stained nucleus and coarse cyanophilic granules.

In view of the fact that these cells can demonstrate certain synthetic activities, it was hoped that specific indexes could be found which would help to clarify the mechanisms involved. To elucidate the morphologic and cytochemical parameters of strain NCTC L 929 fibroblast (*21*), the present study was undertaken on monolayers because this technique facilitated manipulation of the cells. With the vast array of controllable cytochemical procedures now available (*22*), a critical analysis of the metabolic state and function of individual cells is feasible. The present review illustrates some of the methods utilized and points out the variability observed in cellular activity among cells of a clonal strain (*23–27*).

MATERIALS AND METHODS

Strain L cells from an actively growing stock culture [4] were maintained in stationary bottle cultures in medium 199 and 0.5 percent Bactopeptone (*28*) (199P). The cells were scraped from the surface, repeatedly triturated, and pipetted in 0.5 ml aliquots (containing 200,000 cells/ml) into tubes with coverslips. Monolayers were incubated for 48 hours, fixed appropriately, and subsequently stained by a variety of procedures (*27*). For the demonstration of enzyme activity, coverslips were rinsed in balanced salt solution and either placed directly into substrate or frozen at −70° C for 2 minutes before incubation.

OBSERVATIONS

In phase microscopy strain L cells during interphase appeared as stellate, rounded, or spindle-shaped with a prominent, eccentric nucleus containing a number of large pleomorphic nucleoli. The cytoplasm exhibited a vacuolated juxtanuclear zone (*1, 2, 4, 6, 7, 10, 29–33*) capping one side of the nucleus, a granular zone surrounding the nucleus and the juxtanuclear area, and a peripheral nongranular area. Supravital staining with Janus green B demonstrated that the mitochondria were exclusively located in the granular zone (fig. 2). On occasion, giant, sometimes multinuclear, cells were observed in the monolayers.

When the cells are cultivated as a monolayer for 48 hours, they are not distributed in a random manner but appear on the coverslip in colonies. The periodic acid-Schiff (PAS) technique (*34, 35*) selectively stained large, discrete, PAS-positive granules in the juxtanuclear area and a diffuse reticulum in the remainder of the cytoplasm (fig. 3). Such staining was especially obvious in those cells grouped in colonies and less so in single, isolated cells. Similarly, after staining with the colloidal-iron technique (*36*) for acid mucopolysaccharides, it was observed that only those cells concentrated in colonies demonstrated large, blue-staining, colloidal-iron granules in the juxtanuclear area (fig. 4). In addition, colloidal-iron staining, as well as metachromatic staining, was observed at the cell interfaces (fig. 5). This staining often appeared as long intercellular strands reminiscent of the fibers seen in suspension (*15*). Digestion with amylase or ribonuclease and extraction with chloroform-methanol or pyridine did not alter the colloidal-iron stain, but digestion with hyaluronidase for 3 hours completely inhibited the stain. The data suggest the strain L cell as a monolayer was capable of producing an acid mucopolysaccharide that was concentrated in the juxtanuclear area, secreted intercellularly, and dependent upon cell density. Although both Morris (*37*), Berenson and his coworkers (*38*), and others (*39*) biochemically demonstrated the production of acid mucopolysaccharides by fibroblasts *in vitro*, Morris was unable to show cytochemical differences in cells that had produced widely different quantities of acid mucopolysaccharide. Since studies of cell populations cannot discriminate the cellular parameters provided by such morphological and cytochemical data, the use of both biochemical and cytochemical techniques would seem essential to their characterization.

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[4] Generously supplied by Dr. Donald J. Merchant, Department of Bacteriology, The University of Michigan, Ann Arbor, Mich.

Of a similar nature, it was observed that staining with Baker's acid-hematein technique (*40*) demonstrated a surprising variability in phospholipide content among cells. While some cells were negative and others positive, a complete series of intergrades was also apparent (figs. 6 and 7). The variations in staining, however, were not related to cell distribution. The differential content of phospholipide in monolayers may represent cells in different phases of the mitotic cycle, differences in synthetic activity, or a complete gradient of cellular types. Since these cells are in a growth phase comparable to the early log phase of suspension cultures, in a population of cells it appears that metabolic and/or synthetic ability is not incompatible with growth of the population. The variations in activity seen in these monolayers may not be obvious when total cell populations are investigated.

Cells grown *in vitro* provide ideal material for the study of enzymes in general and especially those involved in oxidative metabolism since they do not require freezing and/or fixation, embedding, or sectioning and can be visualized with phase optics just before treatment. Few investigators have used this material. Although Henrichsen (*41*) reported the distribution of alkaline phosphatase, Gropp and Hupe (*42*) and Gropp and Hellweg (*43*) included a study of the distribution of nonspecific esterases, only Wagner *et al* (*44*), using primary explants, did any extensive cytochemical investigations *in vitro*. Kaufman and Hill (*45*) and Ogawa and Okamoto (*46*) recently demonstrated the distribution of succinic dehydrogenase in HeLa and in primary cortical cultures.

Barban and Schulze (*47*), using biochemical procedures, showed that all the enzymes of the tricarboxylic cycle are in the HeLa cell. With contemporary cytochemical techniques in the present study, all the glycolytic and oxidative enzymes studied were found in the strain L cell (*26*). The techniques used depend on the transfer of electrons from a substrate to a tetrazolium salt (Nitro BT) and the resulting precipitation of a colored formazan deposit at the site of enzyme activity. Glucose-6-phosphate dehydrogenase, glycerphosphate dehydrogenase, β-hydroxybutyric dehydrogenase, lactic dehydrogenase (LDH), malic dehydrogenase (MDH), succinic dehydrogenase, diphosphopyridine nucleotide dehydrogenase (DPND), triphosphopyridine nucleotide dehydrogenase (TPND), and cytochrome oxidase have been demonstrated in strain L cell (*26*). Of these, only cytochrome oxidase and succinic dehydrogenase were restricted exclusively to the mitochrondria, TPND was extramitochondrial, and the rest were in both the cytoplasm and mitochondria. While the distribution of enzymes in strain L cells is in good agreement with biochemical studies (*48*), it differs from cytochemical studies on tissue sections (*22*). It was interesting, in view of our findings with the acid-hematein, colloidal-iron, and PAS techniques, that the activity of MDH, LDH, and DPND (figs. 8, 9, and 10) was greatest in cells located in colonies and least in single, isolated cells. As Phillips and Terryberry (*49*) point out, one would expect the subsequent progeny of a single cell to be relatively uniform in appearance and have a relatively stable metabolism. However, they added, respiration in populations of Earle's strain L has been found widely different on various occasions, a fact suggested by our results.

As pointed out, the nucleus of the strain L fibroblast in monolayer is unusually large, ovoid, and eccentrically located. During interphase it contains a loose chromatin network and extremely large pleomorphic nucleoli, which vary in number from cell to cell. Use of the methyl green-pyronin technique (*28*) also provided some interesting morphologic data. As often reported, the nucleolus appears pyroninophilic during interphase, indicative of its ribonucleoprotein content, while the chromatin network (chromonemata) appears dark green to blue green, which implies that deoxyribonucleoprotein is present (*50, 51*). The nucleoli, an important site of nuclear ribonucleoprotein synthesis (*52, 53*), also demonstrates a peripheral rim of methyl green staining (heterochromatin, containing deoxyribonucleoprotein), as has been shown previously (*10, 54–57*), (fig. 11). As the cell enters prophase, the chromatin material condenses (fig. 12) and during the middle of prophase the initial disintegration of the nucleolus was followed by the apparent flowing of pyroninophilic (RNP) material down along the chromonemata (fig. 13). Thus, by late prophase, the nucleolus has disappeared and some of the forming chromosomes are embedded in a matrix of pyronin stain that remains through metaphase (fig. 14). The association of ribonucleoprotein with the mitotic cycle has been demonstrated in a number of cell types *in vitro.*

DISCUSSION

Jacobson and Webb (*58*) and Jacobson (*59*), using the May-Grünwald-Giemsa stain on primary explants of chick osteoblasts and fibroblasts, described the presence of ribonucleoprotein staining of the chromosomes at late prophase, metaphase, and anaphase, as had been pointed out by Brachet (*50*), Frolova (*60*), Ris (*61*), Kaufmann, McDonald, and Gay (*62*), and Turchini (*63*). Jacobson and Webb did not observe the concomitant dissolution of the nucleolus and deposition of ribonucleoprotein on the chromosomes and, in fact, stated that the nucleolus was still intact when the forming chromosomes were already pyroninophilic. Boss (*64*) similarly reported that the chromosomes acquire a ribonucleoprotein matrix before changes in the nucleoli become evident and suggested that the ribonucleoprotein might be derived from scattered fine granules in the nucleoplasm at interphase.

Jacobson and Webb (*58*) also demonstrated that ribonucleoprotein is lost between the separating chromosomes at anaphase. Prescott (*65*) pointed out that since ribonucleoprotein synthesis is entirely nuclear, the transfer of ribonucleoprotein to the cytoplasm could occur through the nuclear membrane (*66*), or by direct release in the interzone between separating chromosomes at anaphase, accompanied by a loss of ribonucleoprotein from the chromosomes (*67–71*). Since there are wide variations in the mitotic cycle within single cultures and even between daughter cells (*10, 72, 73*) and cells have not been successfully cultivated in a synchronous manner, it is not surprising that there is no steady state of ribonucleic acid in a population of cells (*74, 75*).

Morphology cannot be disregarded as an important tool. As one would expect, the standardized conditions of tissue culture do indeed lead to a state of morphological uniformity. And yet, as we have tried to show, differences in population density, mitotic phase, etc., produce heterogeneity in the metabolic and synthetic activity of individual cells. This would suggest the need to consider structure in the characterization of any clonal strain. The biochemical approach necessitates the use of large populations, which tend to mask the intrinsic variation between cells. It is these small differences among cells as they vary in their function that are of increasing importance to biological understanding. In principle, these differences may be investigated by the cytochemical approach. The ultimate goal of histochemistry and cytochemistry is "the quantitative analysis of the cell, its parts and extracellular products *in situ* and in the living cell" (*76*). Cell biology may be on the verge of a renaissance of cytology correlated with a new level of resolution in cytochemical and microchemical procedures (*77, 78*), so that form and function may soon merge. Then, and only then, will we be able to meet the ultimate goals of characterization.

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Plate 10

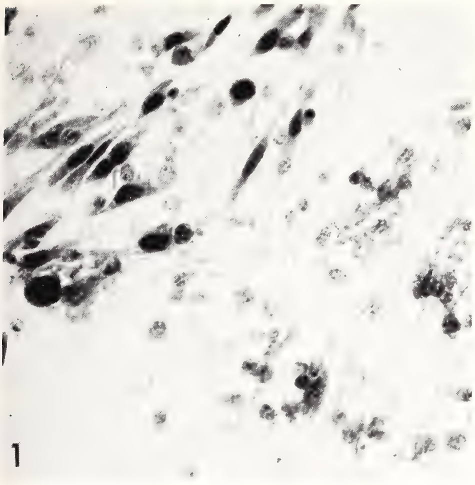
Figure 1.—Membrane formed in suspension culture of strain L fibroblasts. *Note* pleomorphic cells and intercellular fibers. Masson trichrome. × 260

Figure 2.—Strain L fibroblast stained supravitally with Janus green B. *Note* localization of the mitochondria peripheral to clear juxtanuclear area (*arrow*). × 2,400

Figure 3.—Strain L fibroblast stained by the periodic acid-Schiff procedure. *Note* localization of granular material in the juxtanuclear area. × 2,400

Figure 4.—Strain L fibroblast stained by the colloidal-iron procedure. *Note* localization of staining in juxtanuclear area. × 2,400

Figure 5.—Strain L fibroblasts stained by the colloidal-iron procedure. *Note* localization of colloidal-iron stain intercellularly (*arrow*). × 1,200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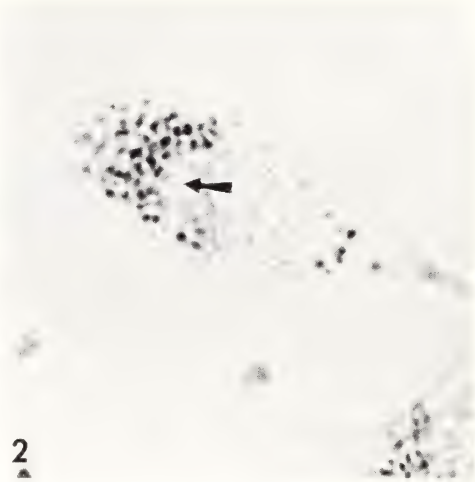
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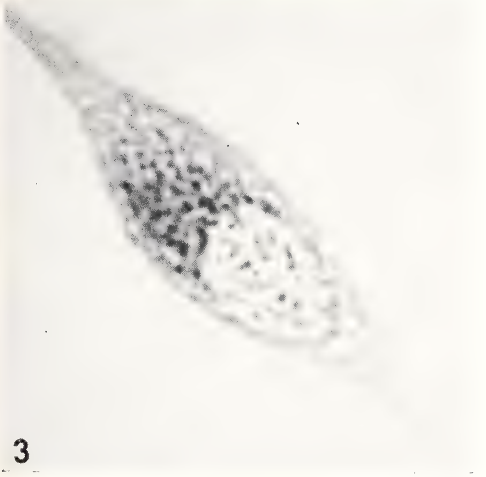
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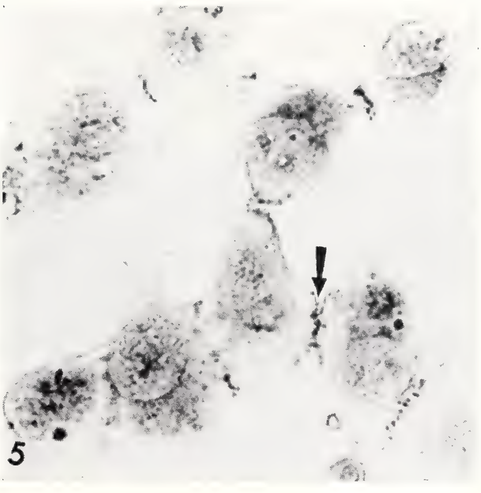
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PLATE 11

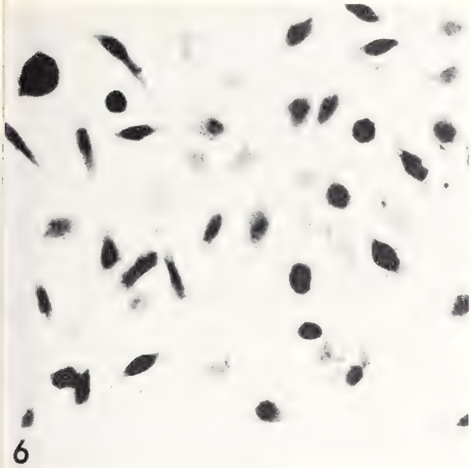
FIGURE 6.—Strain L fibroblasts stained by Baker's acid-hematein procedure. *Note* variability in phospholipide content. × 200

FIGURE 7.—Strain L fibroblasts extracted with pyridine (control) prior to staining with the Baker's acid-hematein procedure. × 260

FIGURE 8.—Strain L fibroblasts demonstrating malic dehydrogenase activity. × 260

FIGURE 9.—Strain L fibroblasts demonstrating diphosphopyridine nucleotide diaphorase activity. × 200

FIGURE 10.—Strain L fibroblasts demonstrating lactic dehydrogenase activity. × 100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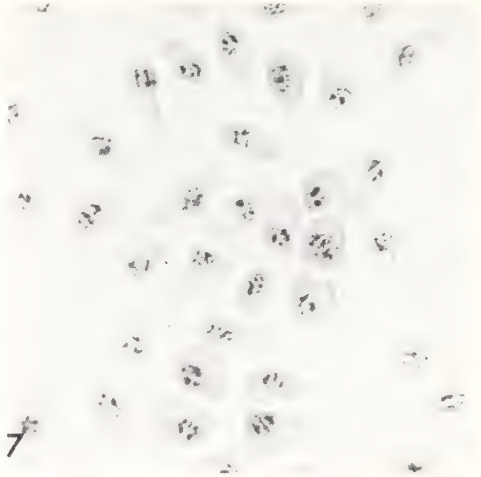
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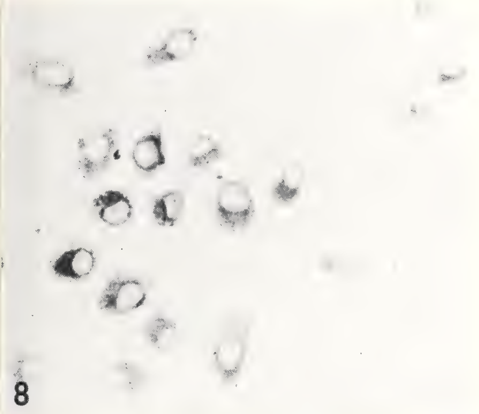
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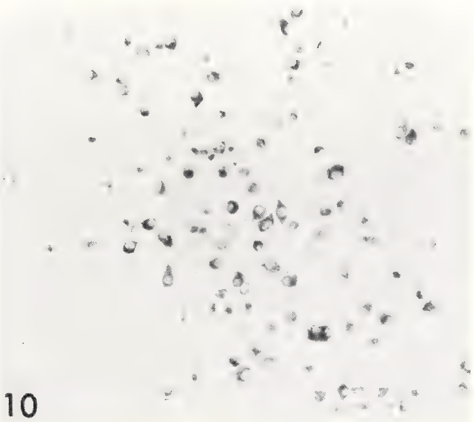
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KAHN, CONKLIN, AND DEWEY

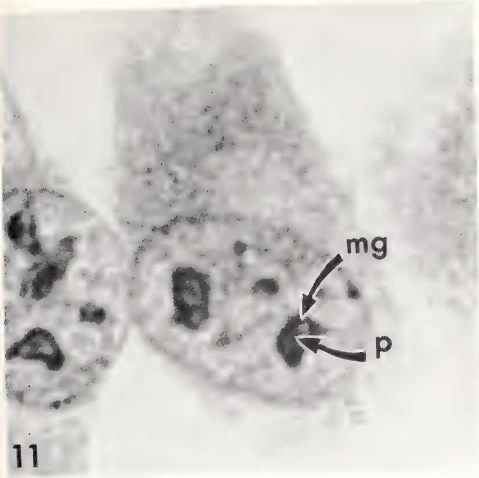
PLATE 12

FIGURE 11.—Strain L fibroblast stained with methyl green (mg) and pyronin (p) during interphase. × 2,400

FIGURE 12.—Strain L fibroblast stained with methyl green (mg) and pyronin (p) during early prophase. *Note* pyroninophilia spreading along the forming chromosomes. × 2,400

FIGURE 13.—Strain L fibroblast stained with methyl green (mg) and pyronin (p) during late prophase. *Note* pyroninophilia along the forming chromosomes. × 2,400

FIGURE 14.—Strain L fibroblast stained with methyl green (mg) and pyronin (p) during metaphase. *Note* pyroninophilic matrix of the chromosome. × 2,400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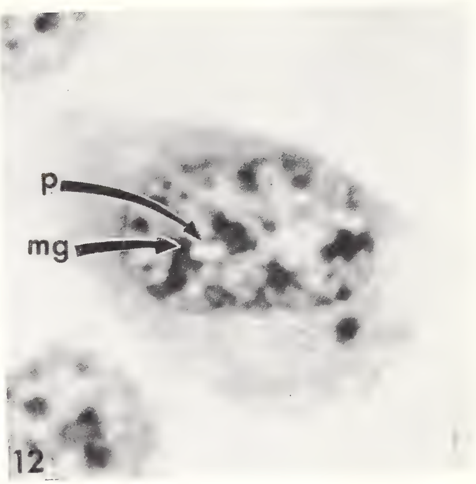
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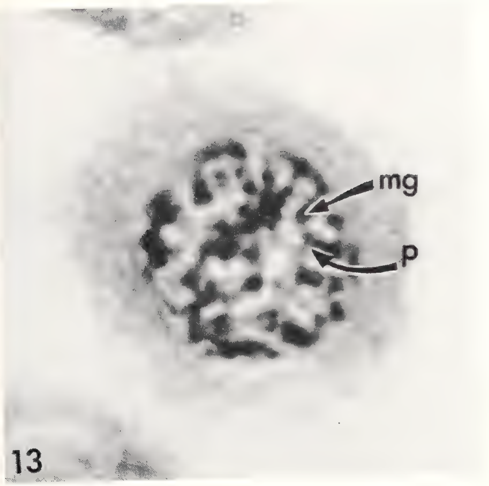
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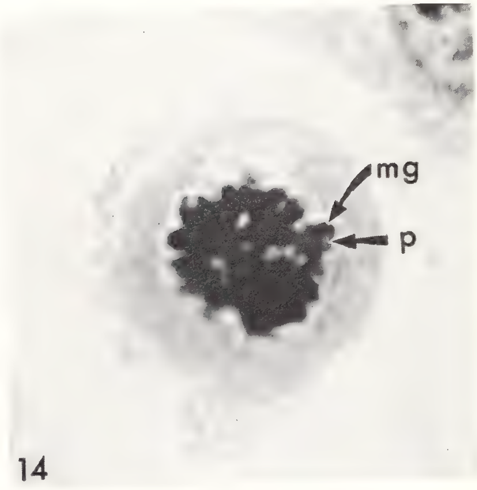
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DISCUSSION

Potentialities of Histochemical Procedures for Enzymes in the Study of the Cell in Culture.[1]

BENITO MONIS,[2] *Department of Pathology, Sinai Hospital of Baltimore, Inc., Baltimore, Maryland*

Reliable histochemical procedures have been developed which permit precise demonstration of some cellular constituents (*1*). These procedures are being applied and it is certain that they will contribute to the integration of morphologic and biochemical information at the cellular level.

The purpose of this brief report is to illustrate the reactivity of some enzymes in cells studied in tissue sections and blood smears and to suggest that histochemical procedures may also be useful for characterizing cultured cells.

Materials and Methods

The various cells described in this report were observed in human tissues and in tissues obtained from rats which were the subject of studies on reparative proliferation (*2*).

Frozen sections of formalin-fixed tissues were used for demonstrating esterase (*3, 4*) and β-D-glucuronidase (*5*).

Fresh-frozen sections were used for the histochemical demonstration of diphosphopyridine nucleotide (DPN) diaphorase (*6*) and aminopeptidase (*7, 8*).

Blood smears, fixed in a mixture of equal parts of absolute ethanol and ether, were assayed for alkaline phosphatase activity (*9*). The source of reagents is mentioned in the articles reporting the methods.

Results

Prostatic epithelium in both man (fig. 1) and rat contains aminopeptidase. The activity is apparent even with very short times of incubation, which suggests that this gland is a rich source of the enzyme.

Fibroblasts in subcutaneous tissue of the adult rat show aminopeptidase activity (fig. 2). Aside from an intensive cytoplasmic reaction, droplets of azo dye are also seen in the cytoplasmic processes and at the cell surfaces. The reaction for aminopeptidase is useful for delineating fibroblasts in tissue sections and in cultures (*10*).

Primitive mesenchymal cells that appear in wounds during the process of healing are very reactive for aminopeptidase (fig. 3). Though large aggregates of copper-azo dye are shown, they do not seem to correspond to organelles. The fibroblasts which are laid down in the wound are also very reactive. Variation of aminopeptidase in experimental wounds and in the serum of rats during wound healing has been reported elsewhere (*2*). The young, newly formed *fibroblasts* growing in a wound contain large amounts of DPN diaphorase (fig. 4). The intense reactivity in these cells reveals tapering processes similar to those found in the mature fibrocyte in subcutaneous tissue of the rat.

Mature macrophages in rat (fig. 5) and in man (fig. 6) react intensely for esterase activity. The reaction is most prominent in the large round macrophage. Frequently these cells reveal pigments such as hemosiderin or carbon.

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[1] Supported in part by grant A-3219 from the National Institutes of Health, Public Health Service.

[2] Acknowledgment is due Mrs. Hannah Wasserkrug and Mrs. Beatrix Carpenter for efficient technical help. Photomicrographs by Mr. Harold Thomas.

Neutrophils in man (fig. 7), as well as in other species, contain abundant alkaline phosphatase.

Mast cells in man show esterase (fig. 8). Convincing evidence that the esterase activity is coextensive with that of the metachromatic component (heparin) was obtained by study of partially degranulated cells.

Plasma cells contain β-D-glucuronidase. Enzymatic activity is frequently found in these cells. Highest reactivity is shown in the juxtanuclear portion of the cytoplasm, the site of the Golgi apparatus (fig. 9).

Discussion

Kahn, Conklin, and Dewey (*11*) have emphasized the usefulness of morphologic and cytochemical procedures for characterizing cells cultured *in vitro*. But there is scant reference to the application of histochemical procedures for enzymes to analysis of cell cultures. And though cultured cells are an ideal population for cytochemical studies, except for the Feulgen procedure (*12, 13*), this approach has been neglected as a tool for the study of the cell in culture (*14*). Reference will be made only to the few reports of results obtained with histochemical procedures for the demonstration of certain hydrolytic and oxidative enzymes.

Cultured chicken monocytes revealed acid phosphatase during their transformation to macrophages (*15*). There are similar observations on the differentiation of normal human monocytes (*16*). In both studies the periodic acid-Schiff procedure showed a glycoprotein at the site of acid-phosphatase activity. The cytoplasm of macrophages and giant cells in tissue sections of chronic inflammatory granulomas reacted likewise (*16, 17*).

Leucine aminopeptidase has been shown in cultures of a line of fibroblasts derived from subcutaneous tissue of the rat (*10*).

A cell line, apparently derived from a giant cell containing no alkaline phosphatase, has been reported to contain little or none of this enzyme (*18*). This may be correlated with the observation of increased alkaline phosphatase in certain cell lines derived from the HeLa carcinoma cell (*19*).

Esterase activity has been shown in cell membranes in macrophages in culture (*20*) and in neoplastic cells in tissue sections (*4*).

Histochemical evidence suggestive of progressive increase of esterase activity of histiocytes during cell maturation has been presented in cultured cells (*20*) and tissue sections (*4*).

Acid phosphatase was demonstrated in HeLa cells by means of an azo-dye method (*21*).

Succinic dehydrogenase has been shown in monolayers of cells grown on coverslips and derived from human malignant and benign tumors and normal tissues (*22*), fibroblasts (*23*), and cerebellum cells (*24*). Negative results for succinic dehydrogenase activity were reported in some cell lines derived from conjunctiva, human kidney and intestine, HeLa, and monkey kidney (*25*).

Diminution of succinic dehydrogenase and cytochrome oxidase activities were found in certain established human lines and in short-term cultures of chick-embryo heart cells by comparison with the corresponding activities in embryonic and adult tissues (*26*).

Succinic dehydrogenase, cytochrome oxidase, DPN diaphorase, and several DPN and triphosphopyridine nucleotide (TPN) linked dehydrogenases were studied in the strain L 929 mouse fibroblast. With the exception of succinic dehydrogenase and cytochrome oxidase, all enzymes had both a mitochondrial and extramitochondrial distribution (*27*).

This survey reveals the limited extent to which available procedures for hydrolytic and oxidative enzymes have been applied to the study of cultured cells. It seems apparent that only in a few instances have attempts been made to use histochemical methods for characterizing cell lines or for tracing processes of cell maturation or cell function.

The existence of several principles and substrates for the demonstration of certain enzymes makes it possible to obtain histochemical evidence of some enzymes with more than one procedure (*1*).

Certain refinements of technique, recently introduced, preserve the integrity of mitochondria which subsequently are clearly visualized by means of their content of succinic dehydrogenase (*28*).

The possibility of accurate intracellular localization should be sought with the aid of cytologic "controls" such as certain classical selective-staining procedures provide.

The paucity of cytochemical studies on cultured cells seems rather surprising since the use of monolayers of cells grown on coverslips makes it possible to apply staining and histochemical procedures without need of previous tissue processing. This may be accomplished with, perhaps, little or no technical modification.

A fruitful approach for the study of cell lines may be a combination of cytochemical and biochemical procedures on intact cells and on cell fractions. Cytochemical techniques may then be applied to tissue and organ cultures with various objectives, some of which are suggested here. Cell lines may be assayed: 1) to establish sequences of enzymatic profiles, 2) to determine histochemical changes in tissues subjected to culture (*29*), and 3) to study the effect on cell constituents, especially enzymes, of controlled variations of media. This approach may also be used to gain insight into mechanisms of action of pharmacologic compounds or pathogenic agents, such as bacterial toxins and viruses (*30*).

Summary

This brief report illustrates several cells observed by means of histochemical procedures in tissue sections and blood smears.

Esterase, aminopeptidase, β-D-glucuronidase, alkaline phosphatase, and DPN diaphorase were demonstrated by histochemical procedures in macrophages, mast cells, plasma cells, neutrophils, and prostatic epithelium in man and fibroblasts and macrophages in rats.

It is suggested that available cytochemical procedures should be applied more extensively to cell lines. This approach may disclose heterogeneity at the cellular level not revealed by biochemical procedures on homogenates. Correlated studies of intact cultured cells and their cellular fractions by means of biochemical and cytochemical procedures also seem desirable. Several possible objectives for the use of cytochemical procedures on cultured cells are suggested.

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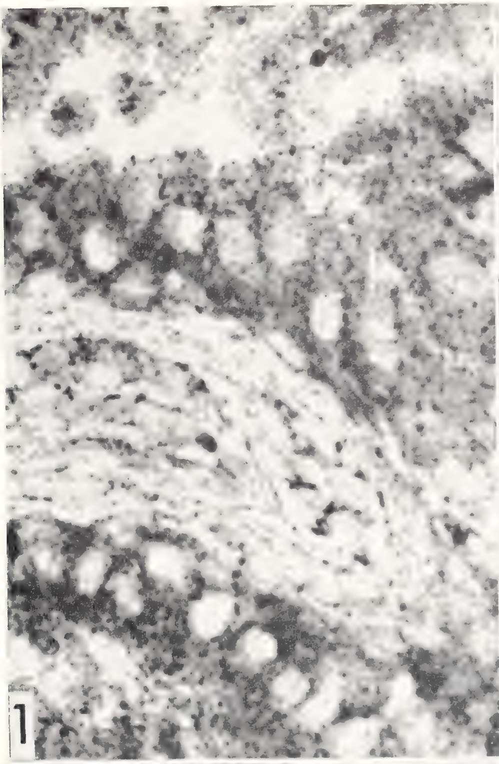
Plate 13

Figure 1.—Prostate, human. Aminopeptidase [DL-alanyl-β-naphthylamide (*31*)]. Glandular epithelium contains large amounts of the product of aminopeptidase activity. *Note* that nuclei are unstained. Fibrocytes in the stroma are also active. Incubation at 37° C for 15 minutes. × 860

Figure 2.—Dermis, adult rat. Aminopeptidase [L-leucyl-*m*-methoxy-β-naphthylamide]. Fibroblasts are intensely active. *Note* processes and droplets of azo dye-copper chelate at cell surfaces. Incubation at 37° C for 2 hours. × 1,000

Figure 3.—Base of surgical wound on skin of a rat, 4 days after wounding. Aminopeptidase [DL-alanyl-β-naphthylamide (*31*)]. *Primitive mesenchymal cells* are intensely active. Incubation at 37° C for 45 minutes (*2*). × 1,700

Figure 4.—Surgical wound in skin of a rat, 4 days after wounding. DPN diaphorase. Fibroblasts showing processes are active in already well-developed granulation tissue. Collagenous bundles that appear refringent are unstained. Incubation at 37° C for 15 minutes. × 700



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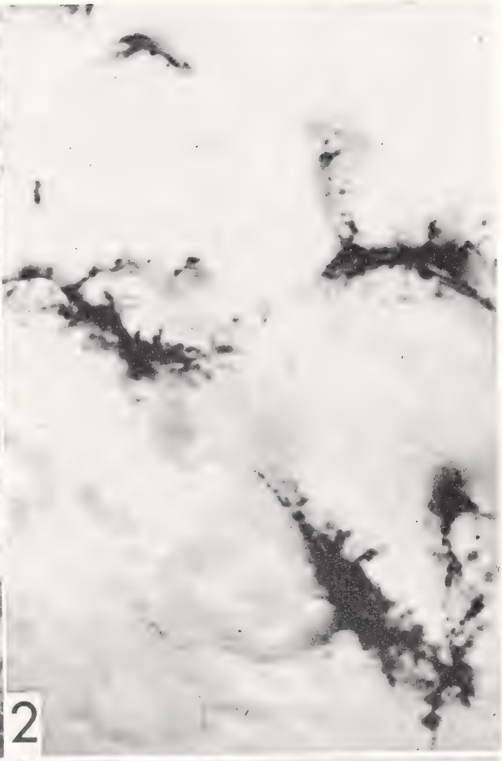

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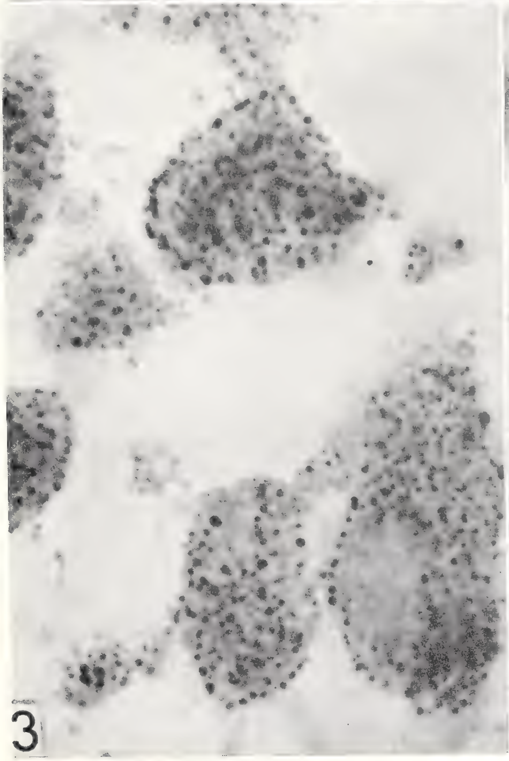

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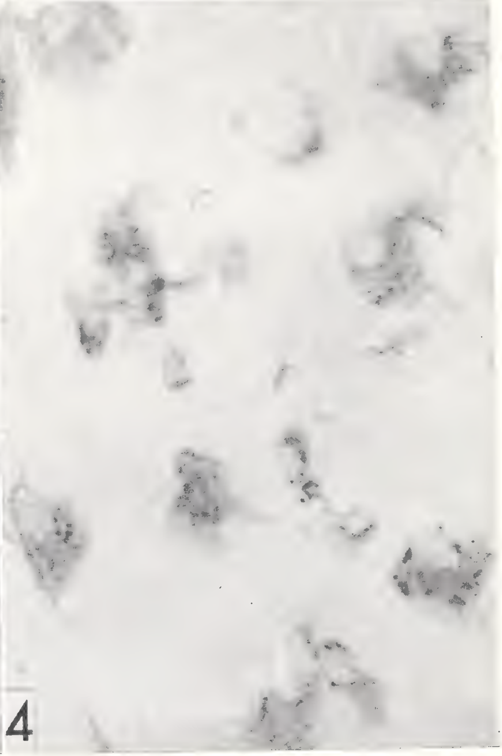

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Plate 14

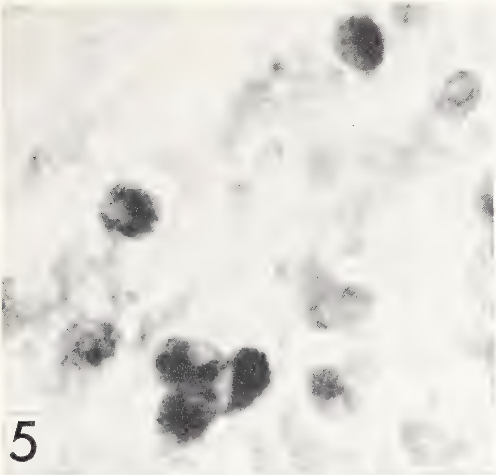
Figure 5.—Surgical wound on skin of a rat, 17 days after wounding. Naphthol AS acetate. Mature macrophages contain intense esterase activity. *Compare* with figure 6. Incubation at room temperature for 15 minutes. × 860

Figure 6.—Macrophage, human. Naphthol AS acetate. Intense esterase activity resulted in heavy deposition of azo-dye pigment. Hemosiderin was demonstrable in this cell in parallel section with the Prussian-blue procedure. Incubation at room temperature for 15 minutes (*4*). × 1,000

Figure 7.—Neutrophil, human, peripheral blood from a patient with agnogenic myeloid metaplasia. Alkaline phosphatase. Naphthol AS phosphate. Cytoplasm is very reactive. Red cells are negative. Incubation at room temperature for 5 minutes. × 1,860

Figure 8.—Mast cell, human. Naphthol AS acetate. The cell contains a few granules of azo dye. The metachromatic reaction showed identical distribution suggestive of close relationship of enzyme with characteristic metachromatic granules. Incubation 15 minutes at room temperature. × 1,400

Figure 9.—Plasma cells, human. 6-Bromo-2-naphthol-β-D-glucuronide. Intense reactivity with highest concentration in a juxtanuclear area of cytoplasm (*32*). Incubation at 37° C for 4 hours. × 1,100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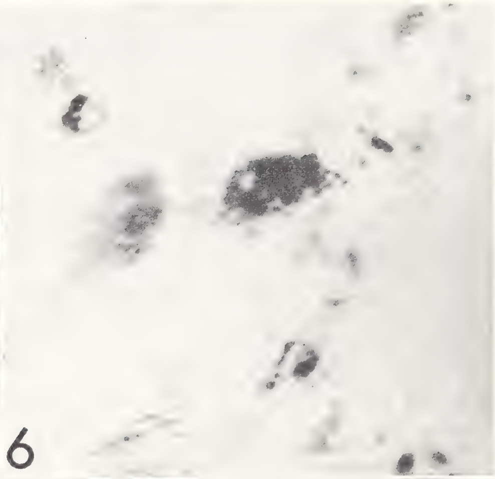
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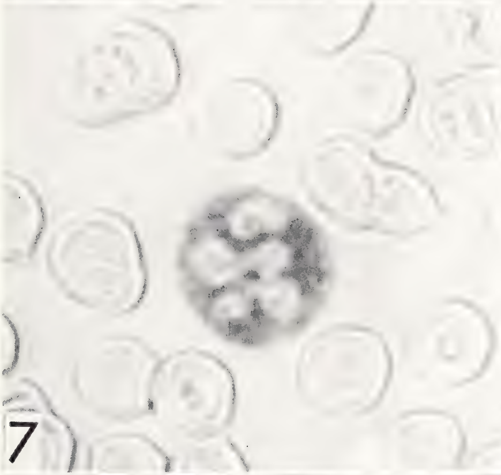
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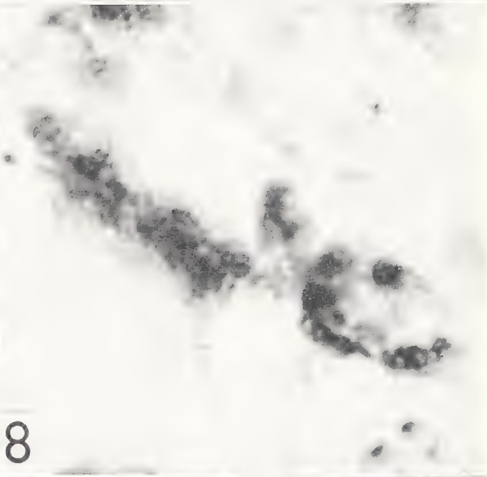
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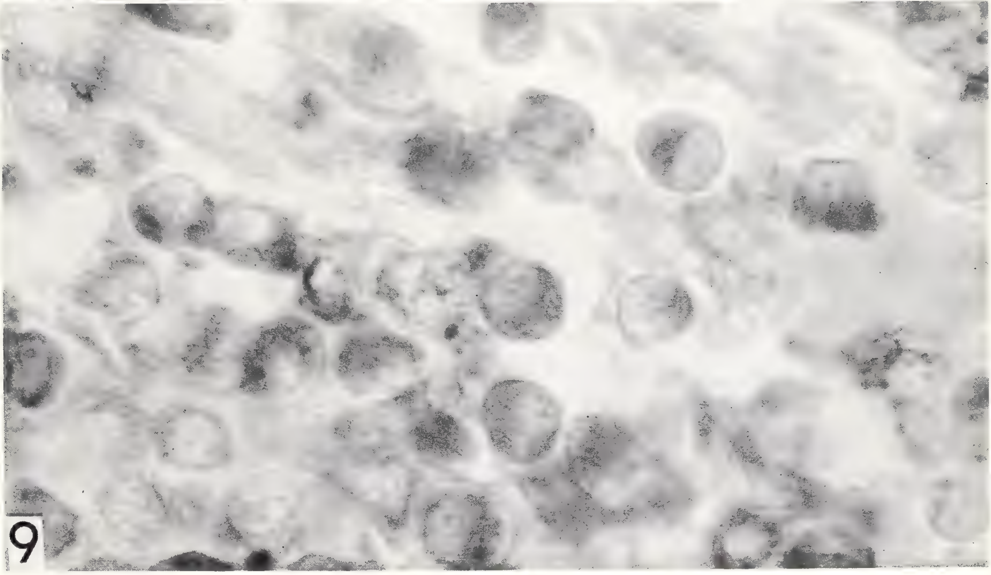
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Characterization of Cells in Tissue Culture[1]

BENTON B. WESTFALL, ***National Cancer Institute,***[2]
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IN ANY study of cells *in vitro* we start with the postulate that living organisms so far as known have only chemical reactions as ultimate causes of all observable events taking place in them. It follows that such observable events as genetic change and enzyme induction or repression must be considered to be mediated through chemical reactions. Many investigators have observed that tissues explanted into culture undergo alteration. This alteration seems to be in the direction of making it possible for the cells to multiply indefinitely and autonomously under certain conditions. Mechanisms through which the changes take place are obscure, and conditions for the most favorable production of such cells are still poorly understood. The biologic importance of these changes for carcinogenesis, as well as the implication for differentiation during early growth, makes study of the alteration of prime importance. It seems that for such studies on mechanisms, cells before and after transformation should be used. The technical difficulties in such studies are still tremendous. Obstacles to be overcome are great, yet progress is being made. There are now in our laboratory some six cell strains of diverse origin growing in defined medium. We have devoted much time and effort to control of the environment for the cells, particularly as represented by removal from the medium of any serum, undefined partial serum digests, or embryo extracts with their possibly deleterious load of virus materials. There are several ways, all closely related, in which cells in culture may be influenced to undergo change:

1. Random mutation with the changed cell favored by the culture conditions to overgrow other cells and by out-reproducing them become the main part of the surviving culture. There are reports in the literature of accidental contamination of human cell cultures with strain L which promptly overgrew the primary culture. This is not a good illustration of what is meant, but is cited simply to indicate that a vigorous strain can dominate the culture.
2. A gradually induced change in the deoxyribonucleic acid (DNA) code with transmission of the change and survival of the progeny.

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[1] Presented at the Syverton Memorial Symposium on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

3. Quantitative changes in the cell components, but not necessarily changes in the qualitative sense.

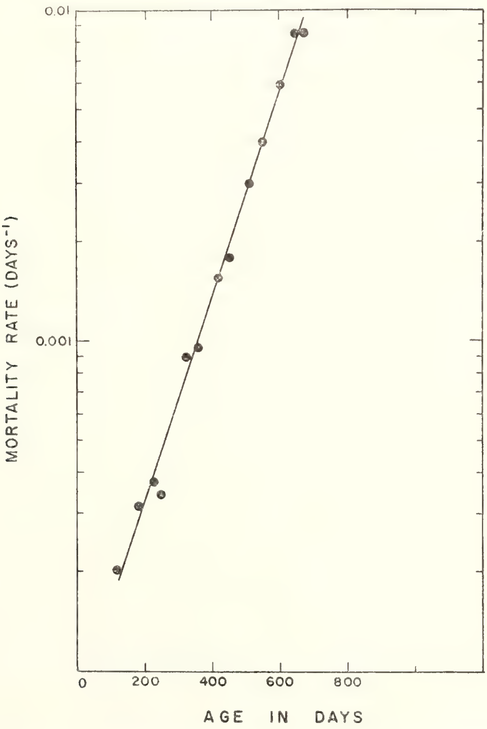
The first of these, random mutation, has always appealed to me largely, I suspect, because randomness seems to be a fundamental property of the organization of the universe. There is general acceptance of the idea that natural selection has operated through the ages. There is not enough evidence to assume that the attributes selected and transmitted had a single origin only. One of the better documented examples of induced changes in cells was reported 5 years ago by Law (*1*) who used the mouse and leukemic lymphoblasts of L1210 leukemia. The cells developed resistance to the antimetabolites for folic acid and for the purine and pyrimidine analogues. Resistance was defined as ability to grow in the presence or absence of the drug. Some strains showed dependence on the drug, as they grew optimally only when it was added. Possible mechanisms considered include:

1. Development of an enzyme with increased ability to convert or inactivate the drug.
2. Quantitative alterations in the substrate-enzyme product system.
3. Development of alternate metabolic pathways for inhibited products.
4. Development of changes in enzyme affinity for the drug compared with the metabolite.
5. Reduction of the transport of an inhibitor to a susceptible enzyme.

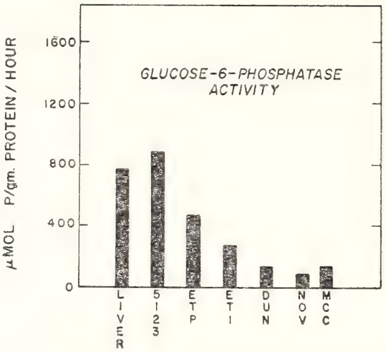
This report is from our studies on cultures derived from a single cell and refers to the third category listed: quantitative, not qualitative, changes in certain enzyme activities of our cells in culture. Before giving these data, however, I want to present data from the work of other investigators which contrast with, or have direct bearing on, the problems under consideration.

Text-figure 1 represents the Gompertz plot (*2*) of the life and death, the biologic summation of the experience of some thousand control mice, from Sacher's report in *Radiology* 5 years ago (*3*). As you probably know, Gompertz showed that this sort of curve held for human populations. This text-figure shows that in an aging population there is a constant increment of death as we proceed along this curve. There is this sort of curve for all populations made up of the aggregate of cells so far examined that we call an individual. As nearly as we can tell at present such considerations do not apply to our cells in culture. Our cells in culture are forever young. It appears that the insults to the individuals which result in the death increments represented in text-figure 1 are not operative in culture in the same way. They may be operative instead in producing certain quantitative changes as previously mentioned. Information is accumulating from related investigative areas which has bearing on some of our problems in tissue culture. There is a considerable program under way on screening and testing antitumor agents, and much of this testing has been carried on with transplanted tumors. This brings us immediately to the problem of whether tumors, such as Sarcoma 180, the Ehrlich ascites tumor, or the Novikoff hepatoma, etc., maintain their reputed characteristics through transplant generations *in vivo*. If testing results are compared from time to time it would appear that they should do so. A relatively large amount of work has been devoted to characteristics of these three tumors grown as transplants in the animal; an entire symposium has been devoted to the Novikoff hepatoma alone (*4*). Now, because many of its enzymes are greatly reduced in quantity or activity, or deleted from the hepatoma mentioned, it is becoming fashionable to suggest that it was derived not from parenchymal epithelium, as originally reported, but from bile-duct cells (*5*). The work in tissue culture of Paul and Pearson (*6*), Perske *et al.* (*7*), and Eagle *et al.* (*8*) has led Weinhouse (*9*) to suggest that early in the cultivation of liver cells *in vitro*, hepatic characteristics disappear. This suggestion points up the fact that we may need a new definition of what the term "hepatic" means. This aspect is emphasized by recent work from Dr. Van R. Potter's laboratory (*10*, *11*).

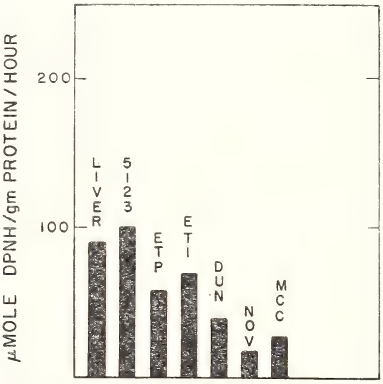
Text-figure 2 is a summary of Dr. Van R. Potter's recent examination of various hepatomas for glucose-6-phosphatase activity. Note that only one of these, Hepatoma 5123, bears much resemblance quantitatively to liver. Text-figure 3 gives similar data for glutamate dehydrogenase



TEXT-FIGURE 1.—Age-specific mortality rates for mice (*3*).



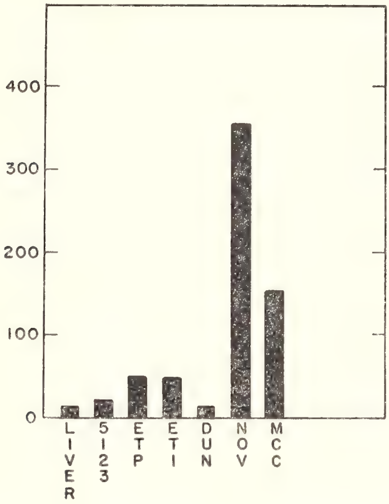
Text-figure 2.—Glucose-6-phosphatase activity of liver and hepatomas.



Text-figure 3.—Glutamate dehydrogenase activity of liver and hepatomas.

activity of the same tissues. Again there is much variation, with Hepatoma 5123 as the only tissue resembling liver. Text-figure 4 shows the activities for the enzyme deoxycytidylate deaminase; again liver and Hepatoma 5123 resemble each other, but in this instance their activities are much lower than the values for the McCoy and Novikoff hepatomas. Because of the great variation in enzyme activities in these tissues, the present trend is to question the cellular origin of the different tumors. There is, however, little evidence one way or another to support the hypothesis that some are of bile-duct as opposed to parenchymal-epithelial origin.

In a recent paper, Trams *et al.* (*12*) found that in the Sprague-Dawley rat treated with the carcinogen *N*-2-fluorenylacetamide there was an increase in the activity of three liver enzymes examined: glutathione reductase, glucuronyl transferase, and uridinediphosphateglucose dehydrogenase. Much work has accumulated in the literature on enzymes in tumors, particularly transplanted ones. Both primary and transplanted tumors appear to be poorly suited to the study of the mechanism of carcinogenesis as far as enzymatic activity is concerned. Studies on them contribute to knowledge of the state, not the process. They represent the status after the fact. What is needed is study during the process.



TEXT-FIGURE 4.—Deoxycytidylate deaminase activity.

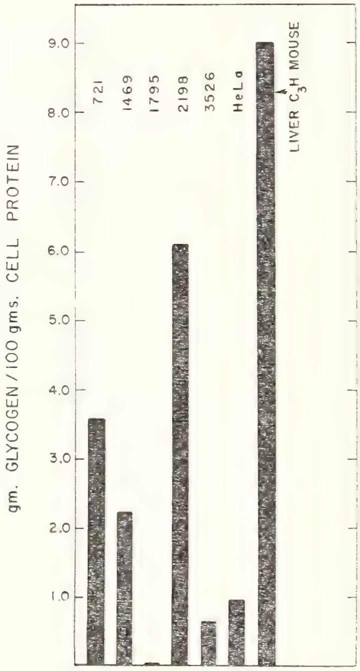
I cite these findings as background for some results with cells from our laboratories which were grown for long periods in culture. Had there been reports of such results in the literature 5 years ago, when our work on clonal markers for cells in culture was started, it might have saved us considerable concern over some unexpected findings.

Our original venture with a study of certain markers for cells in culture had three origins:

1. To aid in identifying cells as to tissue of origin, *e.g.*, epidermis, liver parenchyma, connective tissue, etc.
2. To ascertain whether certain properties stabilized or varied as the cells were carried in culture.
3. To ascertain whether different media or growth conditions had any measurable effect on certain attributes of the cell.

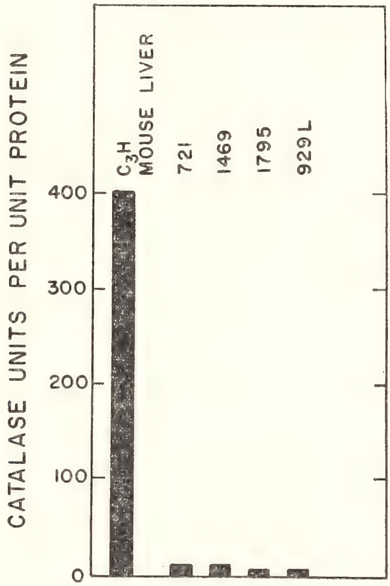
When we first started work on biochemical markers, glycogen storage was the initial capacity studied. We began with this because a very interesting strain had been started in the laboratory from the parenchymal-epithelial cells of the young mouse. The great difficulties involved in the establishment of liver epithelial cells have caused many investigators to regard the growing of liver epithelium in culture as an insoluble problem. The strain of cells, designated NCTC 721, stored glycogen and still does after 12 years in culture, as does clonal strain, NCTC 1469, from strain 721. The findings on several cell strains from the mouse, human, and monkey, are shown in text-figure 5. The finding of glycogen in two of the three strains of mouse liver origin, in the strain of monkey kidney origin, and in the two strains of human origin implies that the enzyme chain concerned in deposition of glycogen in these cells is still intact. In addition to the strains listed in this text-figure, ten fibroblast strains derived from the mouse were examined; all were negative for glycogen. Text-figure 6 depicts the catalase activity of the several cell strains.

Catalase activity has almost disappeared. In contrast to this are the results for β-glucuronidase activity from the studies of Kuff and Evans (*13*). Text-figure 7 shows that all the long-term strains have much more β-glucuronidase activity than liver from the mouse strain of origin of the cultured cells. One of the interesting aspects in studies of this enzyme is that there are mouse strain differences with respect to activity of the enzyme in the liver. There is more than 100 percent difference in strain NCTC L 929 and its derived strain NCTC 2071, grown in the absence of any added protein or unknown peptide material in the medium. A possible explanation of this is that the serum may furnish an inhibitor for the enzyme in the case of strain L 929. Data of Adebonojo *et al.* (*14*) summarize the behavior of our longest term strain, L 929, in the response of four enzymes to anaerobiosis (text-fig. 8). That cytochrome oxidase activity could be lowered, and at the same time two of the glycolytic enzymes increased, is in agreement with the idea that cells in culture may readily change their enzyme activities in response to environment. These results are also in agreement with those of Ephrussi *et al.* (*15*) who, working with yeast, found that changes in enzyme activity brought about by anaerobiosis were reversible. Obviously there are limits to such a generalization. Text-figure 9 summarizes our findings on arginase activity of the cells in long-term culture. The activities have been maintained for several years (*16*).

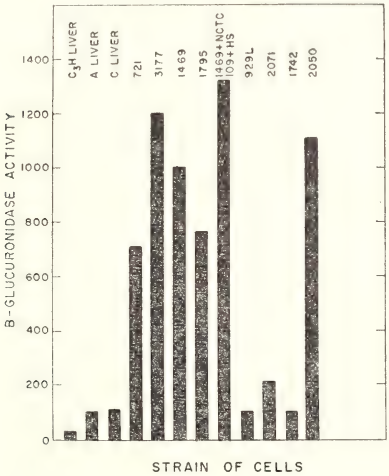


TEXT-FIGURE 5.—Glycogen storage in mouse liver and in cell strains from mouse, human, and monkey.

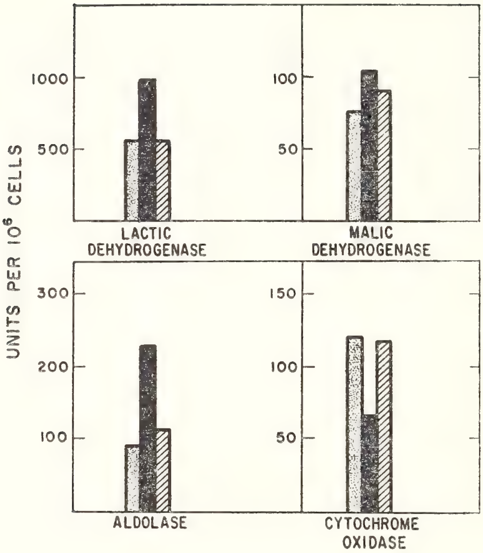
The events leading to the augmentation of activity in the two fibroblast strains remain obscure. When those strains producing tumors are injected into animals, the tumors arising from the injected cells maintain the relatively high or low arginase activity of the cultured cells. At the stage of their existence when they were first sampled, the arginase activity had apparently reached a relatively stable state. The three strains presumably from epithelial cells of the mouse liver retained high arginase activity; eight of the ten mouse fibroblast strains examined at



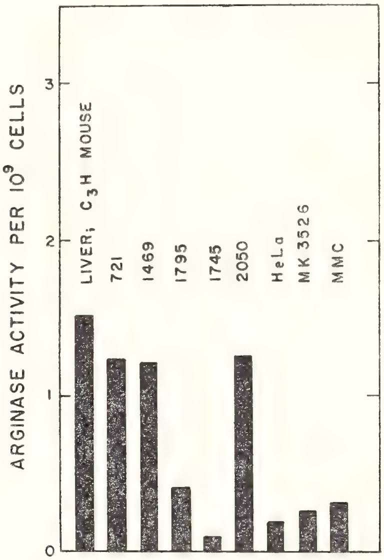
Text-figure 6.—Catalase activity of several cell strains.



Text-figure 7.—β-Glucuronidase activity in mouse liver and in cultured cell strains.



Text-figure 8.—Enzyme activity in strain L 929 cells grown aerobically and anaerobically.

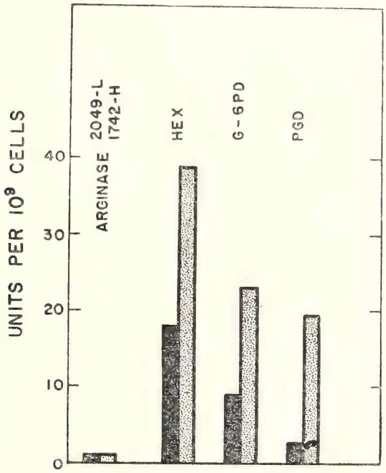


Text-figure 9.—Arginase activity of mouse liver and of cells in long-term culture.

this time had barely detectable activity of the enzyme, but the two cloned strains, NCTC 1745 and NCTC 2050, had activity but differed from each other. The human strain HeLa from uterine tissue retained some activity in keeping with the presumed tissue of origin. The cloned monkey kidney strain, NCTC 3526, had activity similar to kidney tissue from the monkey, as reported in the literature. A mouse mammary-carcinoma strain was also active. Previously we had reported that three clones derived from mouse mammary carcinoma lacked arginase activity. Those clones on injection into the mouse strain of origin gave sarcomas rather than carcinomas so that it appears that during cultivation of the mixed tissue from the original carcinoma the fibroblast cells were selected for cloning. A strain of human epidermis had almost no activity. The long-term mouse tissue strains were examined by Tomkins for steroid reductase activity (*17*). Liver epithelial cells are reputed to be the only tissue of the mouse that has the four enzymes. All the liver cultures had some activity, though low compared to the fresh liver tissue. Incidentally, Dr. Tomkins found that with these four reductases there were strain differences in mice, the C3H mouse having lower activity of the liver enzymes than other strains. Like the β-glucuronidase activity previously mentioned, these activities appear to be under genetic control. The other mouse cell strains were lacking in activity. Scott *et al.* examined the clonal strains from the high tumor- and low tumor-producing lines of fibroblasts for activity of three enzymes, and these are shown in text-figure 10. The high-line clone had by far the greatest activity (*18*).

Obviously much remains to be done, especially during the very early time after the cells have been taken from the animal into culture. Techniques for working with single cells as they adapt to growth in culture would be advantageous.

One final word about the characterization of the cells from culture: Our primary problem is that of cancer. Many of the cell strains after long cultivation *in vitro* give rise to tumors when injected into animals. What I regard as a highly significant experiment was recently reported by Sanford *et al.* (*19*): Polyoma virus was grown in mouse salivary-gland cultures and these infected cells were periodically injected into mice to see if they were undergoing malignant transformation. The control portion of the same strain of cells, uninfected, was likewise injected from



TEXT-FIGURE 10.—Activity of high- and low-line strains for hexokinase, glucose-6-phosphate dehydrogenase, and phosphogluconate dehydrogenase.

time to time. The problem arises as to how one would know, in the event that the infected cultures gave rise to tumors, whether the tumor came from the cells grown in culture or from virus in the cells transforming the cells of the host mouse to tumor. In the field of virology, there have been reports of malignant transformation with inadequate information on this point, but there is a technique enabling one to answer this question: Dr. Sanford used a hybrid mouse of the F_1 generation-cross of the C3H line on C as host to receive the cells. If the tumors that arose in this hybrid generation were then transplanted back into the parent strains, if they came from the C3H cells *in vitro*, the tumors would grow in the C3H hybrid, in the C3H parent, but not in the C parent. If the tumors came from the virus transforming the cells of the hybrid to tumor, they would grow in the C3H × C hybrid, but not in the C3H or C parent. However, Dr. Sanford found that the cells grew in C3H × C hybrid, in the C3H host, not in the other. The control, uninfected culture gave no tumor at 4 months, but the cell strains were carried in culture and periodically injected. By 10 months, both infected and uninfected lines gave rise to sarcomas on injection. What does this experiment say to us? Barring the possibility that there may have been present a distinct unknown virus in the control cultures, to a simple-minded chemist what it seems to say is that the virus in this instance had little to do with the tumor formation. It merely hastened the event.

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DISCUSSION

C. William Castor, *University of Michigan, Ann Arbor, Michigan*

Dr. Westfall's description of comparative enzyme activities in different cell lines illustrates an important approach to the problem of cellular alteration, with obvious bearing on the mystery of malignancy. While delineation of quantitative changes in the specific enzymatic activities of cells in culture is a legitimate focus for those concerned with neoplasia, there is no less reason to hope that cell-culture methodology can contribute useful information concerning the cellular basis of degenerative and chronic inflammatory processes. In these latter situations it may be to the investigator's advantage to study cellular derivatives that are closely related to the original tissue. Measurement of enzymatic activities might be a useful method for "monitoring" newly derived cell lines for "normality."

Dr. Westfall's data indicate that quantitative differences exist in specific enzymatic activities when different cell lines are compared. While the observed differences are incontestable, we have wondered how to interpret them. Are these differences a reflection of the ease of extraction of enzymes from different cell lines; are they due to the presence of inhibitors or activating substances in the crude or partially purified extracts, or are these different enzymatic activities a reversible reflection of the cells' adaptation to an artificial environment? This latter situation was illustrated by Dr. Westfall when he cited the variation in oxidative enzymes of the strain L cell in response to lack of oxygen. One wonders how general this phenomenon may be, and how concerned we really ought to be when we observe quantitative alterations in the enzymatic activities of primary culture material.

Some cell functions stand out as obvious "handles" for cell characterization. Evidence for glycogen synthesis, as described earlier, would fit in this category. We might add mucopolysaccharide synthesis in the case of connective-tissue cells and certain other cell types. These materials, in the main, are elaborated into the media and may be measured as an additional parameter for cell characterization.

Besides looking at the cell's enzymatic complement and special products, we should remember that biochemical tools are available for exploring differences in structural proteins and lipides, as well as nucleic acids. While some chemical information is forthcoming concerning the chemical composition of unaltered cells of primary culture status, we are limited in what we can do—not so much by the shortcomings of biochemical methodology as by our inability as cell culturists to rapidly produce multigram quantities of unaltered cells.

Session III

Characterization of Cell Strains

Chairman: Hilary Koprowski, *M.D., Wistar Institute, Philadelphia, Pennsylvania*

Characterization of Cell Strains by Viral Susceptibility [1, 2]

ROBERT N. HULL *and* O. JEANNETTE TRITCH,
Lilly Research Laboratories, Indianapolis, Indiana

WE HAVE developed and maintained continuous cell strains of the monolayer type in our laboratory for over 10 years. During this time we have been constantly aware of the problem of individual strain identification and have attempted to solve it by immunological techniques and by differences in virus susceptibility. The immunological studies were done some time ago and were somewhat crude compared to similar methods now appearing in the literature. Antiserums were prepared against a number of cell strains and were assayed, with cytotoxicity as an indicator of activity. Cell strains originating from species as widely separated as man and mouse were distinguishable with these serums, but more closely related species such as man and monkey showed extensive cross-reactions. It also was not possible to distinguish between two cell strains of the same species of origin. To our knowledge none of the newer immunological approaches could be used to identify individual cell strains of the same species. The possibility of developing a system of strain-specific virus sensitivity patterns for the various cell strains in our collection was undertaken, and the results are described in this report.

MATERIALS AND METHODS

Cell strains.—The cell strains included in this study are listed in table 1 and were obtained from the sources indicated. The LLC strains (Lilly Laboratory Culture) were established in our laboratories. All strains except LLC-MK_2 (NCTC 3196) were grown on medium 199 plus 1 or 5 percent horse serum. Strain LLC-MK_2 (NCTC 3196) was grown on the chemically defined medium NCTC 109 (*1*) without added protein.

Viruses.—Table 2 lists the viruses and indicates their source, passage histories, and strain designations. All were used as a crude harvest from tissue culture.

Procedures.—Virus growth in a cell strain was recorded as positive only when the specific cytopathogenic effect (CPE) occurred consistently

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[1] Presented at the Syverton Memorial Symposium on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

[2] Data included in this paper were previously presented at the Tenth International Congress for Cell Biology and were published under a different title in the French Journal, Path. et. Biol. 9: 722-727, 1961.

TABLE 1.—Cell strain data

Cell strains	Source	Species of origin	Time in our laboratory (years)
HeLa	Microbiological Associates, Inc.	Human	7
J-96II	Osgood	Human	6
AV-2	Puck	Human	4½
NCTC 2414	Earle	Human	5
HEp-2	Moore	Human	4
Det-6	Stulberg	Human	4
ERK-1	Wistar Institute	Rabbit	4
CH	Salk	Monkey	4½
LLC-MK$_1$	Lilly Laboratories	Monkey	6½
LLC-MK$_2$	Lilly Laboratories	Monkey	6½
LLC-MK$_2$ (NCTC 3196)	Evans	Monkey	2¼
NCTC 1742	Sanford	Mouse	5
NCTC 2049	Sanford	Mouse	5
LLC-Me$_1$	Lilly Laboratories	Monkey	7½
LLC-He$_1$	Lilly Laboratories	Human	7
LLC-MS$_{180}$	Lilly Laboratories	Mouse	7¼
LLC-AS$_{180}$	Lilly Laboratories	Mouse	6½
LLC-M$_1$	Lilly Laboratories	Mouse	10
LLC-M$_3$	Lilly Laboratories	Mouse	6½
LLC-WRC$_{256}$	Lilly Laboratories	Rat	6¼
LLC-PK$_1$	Lilly Laboratories	Pig	2½
LLC-RK$_1$	Lilly Laboratories	Rabbit	1¾

TABLE 2.—Virus data

Virus	Source	Passage history	Strain designation
Poliovirus I	Connaught Laboratories	15 pMK*	Mahoney
S.V.$_2$	Monkey kidney tissue	9 pMK, 9 LLC-MK$_2$	2382
S.V.$_6$	Monkey kidney tissue	8 pMK	1631
Adenovirus-2	Huebner, National Institutes of Health (NIH)	9 pMK	?
ECHO-9	Sabin, Univ. Cincinnati	10 pMK	Hill
S.V.$_{28}$	Monkey CNS tissue	4 pMK, 7 LLC-MK$_2$	9128
S.V.$_{34}$	Monkey CNS tissue	2 pMK, 7 LLC-MK$_2$	A7644
S.V.$_{25}$	Monkey kidney tissue	5 pMK	8045 2WN
S.V.$_4$	Monkey kidney tissue	8 pMK, 4 LLC-MK$_2$	1715 UWB
Swine influenza	NIH	38 Mouse, 34 egg, 3 pMK	1976
Vaccinia	Human vaccination lesion	2 pCK,† 1 pCE,‡ 2 LLC-Me$_1$, 32 pRK§	2368
Coxsackie B-5	Huebner, NIH	1 pMK (in our laboratory)	30601
Herpes simplex	Heilman, Mayo Clinic	1 Mouse brain, 13 pRK, 1 CAM, 5 pRK	1814
B virus	Rabbit brain	13 pMK, 36 pRK	1418
SE polyoma	Stewart, NIH	No passages in our laboratory	2510

*pMK=primary monkey kidney.

† pCK=primary calf kidney.

‡ pCE=primary chick embryo.

§ pRK=primary rabbit kidney.

through 3 or more serial passages of the virus in the cell strain. At each passage the virus was diluted so that the accumulated dilution in the final passage was well beyond the titer of the initial virus seed. In most instances the virus also was titered in the final passage. The experimental procedure was somewhat like that used by the analytical chemist, except the presence or absence of a CPE was looked for rather than a precipitate. All cell strains were first tested for sensitivity to one virus and thus divided into one group of positive strains and one group of negative strains in respect to their susceptibility. Each group of cell strains was then tested with other viruses and further subdivided into smaller groups until eventually the groups were reduced to single strains.

RESULTS

The fact that poliovirus was infectious only for primate cell cultures was used to make the first separation of this group of 24 cultures. Text-figure 1 indicates the results of this test. Measles virus could have been used as well as poliovirus at this stage, but its CPE was not as extensive as that produced by poliovirus, and it was also a less stable virus. Each strain reacted as expected except the embryonic rabbit kidney strain ERK-1, which was poliovirus-positive, and the human- and monkey-embryo strains LLC-He$_1$ and LLC-Me$_1$, which were not susceptible to the virus.

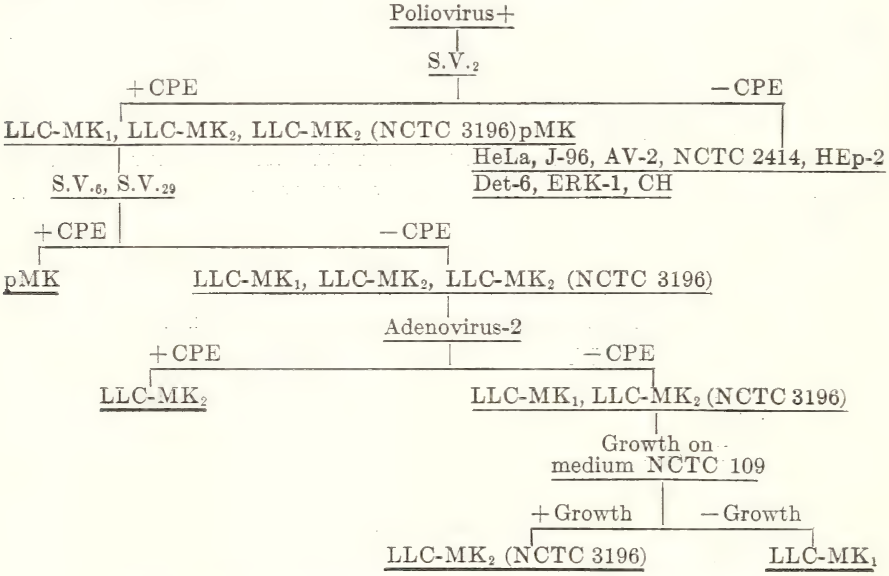
Poliovirus I

+ Cytopathogenic effect (CPE)	− Cytopathogenic effect (CPE)
HeLa	NCTC 1742
J-96	NCTC 2049
AV-2	LLC-Me$_1$
NCTC 2414	LLC-He$_1$
HEp-2	LLC-MS$_{180}$
Det-6	LLC-AS$_{180}$
ERK-1	LLC-M$_1$
CH	LLC-M$_3$
LLC-MK$_1$	LLC-WRC$_{256}$
LLC-MK$_2$	LLC-PK$_1$
LLC-MK$_2$ (NCTC 3196)	LLC-RK$_1$
pMK	pRK

TEXT-FIGURE 1.—Susceptibility of cell strains to poliovirus.

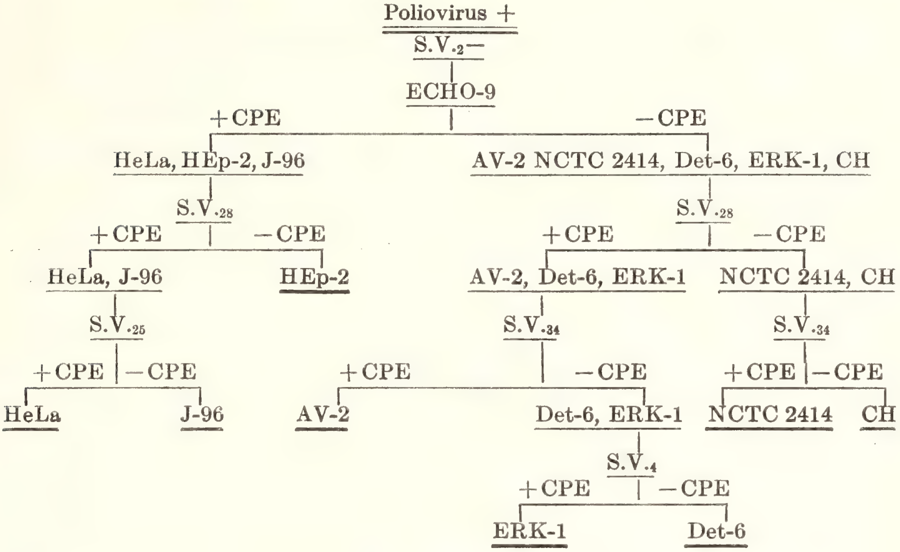
In the poliovirus-positive group, the strains of monkey kidney origin were separated from the others by their ability to grow the simian enterovirus S.V.$_2$ (*2*). Sensitivity to this virus, or to the other members of the (CPE) group II simian viruses, appeared to be highly specific for cells of rhesus or cynomolgus monkeys, since this virus could not be grown in any of the human cell strains or in kidney cells from several other species of monkeys including the African green monkey (*Cercopithecus aethiops*).

Text-figure 2 shows the separation of strains obtained with S.V.$_2$ and the further characterization of this subgroup. S.V.$_6$, also a simian enterovirus, was used to distinguish primary rhesus monkey kidney cells (pMK) from the established strains of this cell type. This virus could not be cultivated even in second-generation monkey kidney cells. Surprisingly, adenovirus-2 grew only in strain LLC-MK$_2$ and served as a reliable tool for its separation from the other 2 monkey kidney cell strains. Further separation of strains LLC-MK$_1$ and LLC-MK$_2$ (NCTC 3196) by virus sensitivity was not possible at this time, though a marked quantitative difference was seen in respect to their sensitivity to a particular strain of ECHO-11 virus. Strain LLC-MK$_1$ was readily infected by this virus, while growth in strain LLC-MK$_2$ (NCTC 3196) was obtained only after a period of adaption. Thus at this time, since qualitative differences in virus sensitivity were desired, the 2 strains could be distinguished from each other only through the ability of strain LLC-MK$_2$ (NCTC 3196) to proliferate on the chemically defined medium NCTC 109.


Poliovirus+
S.V.$_2$
+CPE
−CPE
LLC-MK$_1$, LLC-MK$_2$, LLC-MK$_2$ (NCTC 3196)pMK
HeLa, J-96, AV-2, NCTC 2414, HEp-2
Det-6, ERK-1, CH
S.V.$_6$, S.V.$_{29}$
+CPE
−CPE
pMK
LLC-MK$_1$, LLC-MK$_2$, LLC-MK$_2$ (NCTC 3196)
Adenovirus-2
+CPE
−CPE
LLC-MK$_2$
LLC-MK$_1$, LLC-MK$_2$ (NCTC 3196)
Growth on
medium NCTC 109
+Growth
−Growth
LLC-MK$_2$ (NCTC 3196)
LLC-MK$_1$

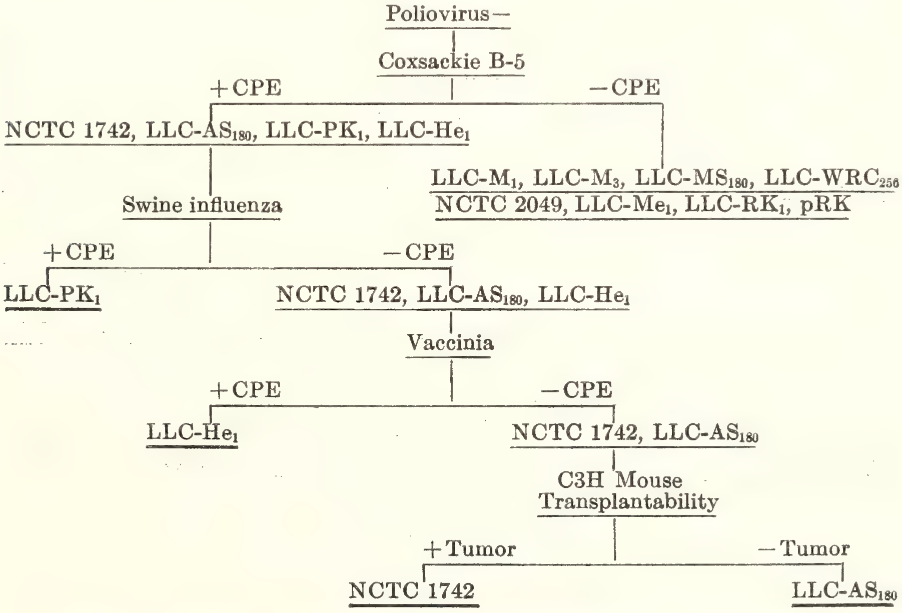
TEXT-FIGURE 2.—Characterization of monkey kidney cell strains.

The poliovirus-positive, S.V.$_2$-negative group of cell strains, which included the strains of human origin plus ERK-1 and CH, was first subdivided in respect to their sensitivity to ECHO-9 virus. These data and the subsequent separation of this group of cells are given in text-figure 3. Many viruses were studied before those listed in the table were finally selected. The separation of Detroit-6 (Det-6) from ERK-1 proved the most difficult. Both strains were susceptible to S.V.$_5$, S.V.$_{12}$, S.V.$_{28}$, Coxsackie B-5, vaccinia, Herpes simplex, B virus, HA-1, measles, and CA, while both were resistant to S.V.$_{25}$, S.V.$_{27}$, S.V.$_{34}$, and ECHO-9. As noted, differences in susceptibility to S.V.$_4$ finally solved this problem.


Poliovirus +
S.V.₂−
ECHO-9
+CPE
−CPE
HeLa, HEp-2, J-96
AV-2 NCTC 2414, Det-6, ERK-1, CH
S.V.₂₈
+CPE
−CPE
HeLa, J-96
HEp-2
S.V.₂₈
+CPE
−CPE
AV-2, Det-6, ERK-1
NCTC 2414, CH
S.V.₂₅
+CPE
−CPE
HeLa
J-96
S.V.₃₄
+CPE
−CPE
AV-2
Det-6, ERK-1
S.V.₃₄
+CPE
−CPE
NCTC 2414
CH
S.V.₄
+CPE
−CPE
ERK-1
Det-6

TEXT-FIGURE 3.—Characterization of human, ERK-1, and CH cell strains.

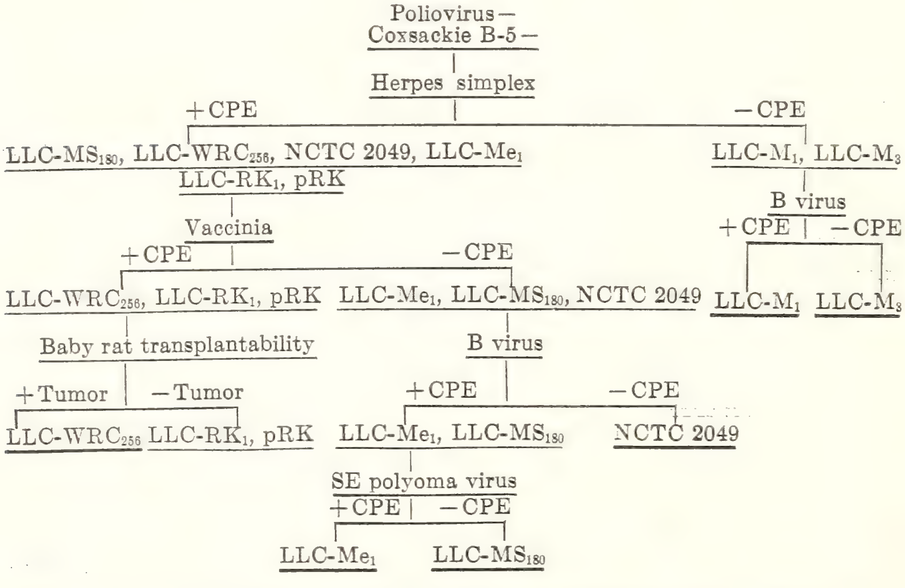
The cell strains in the poliovirus-negative group proved more difficult to separate since fewer viruses were found that could be propagated in these mostly rodent cell strains. The results in text-figure 4 indicate that Coxsackie B-5 virus can be cultivated in 4 of these strains, while 7 others, plus primary rabbit kidney cells (pRK), resist infection by this virus. Of the Coxsackie B-5 positive strains, the pig kidney strain


Poliovirus−
Coxsackie B-5
+CPE
−CPE
NCTC 1742, LLC-AS₁₈₀, LLC-PK₁, LLC-He₁
LLC-M₁, LLC-M₃, LLC-MS₁₈₀, LLC-WRC₂₅₆
NCTC 2049, LLC-Me₁, LLC-RK₁, pRK
Swine influenza
+CPE
−CPE
LLC-PK₁
NCTC 1742, LLC-AS₁₈₀, LLC-He₁
Vaccinia
+CPE
−CPE
LLC-He₁
NCTC 1742, LLC-AS₁₈₀
C3H Mouse Transplantability
+Tumor
−Tumor
NCTC 1742
LLC-AS₁₈₀

TEXT-FIGURE 4.—Characterization of poliovirus-negative cell strains.

LLC-PK$_1$ was easily separated through its susceptibility to the swine strain of influenza virus. The human embryo cell LLC-He$_1$ was readily infected and destroyed by vaccinia virus, while the 2 mouse cell strains NCTC 1742 and LLC-AS$_{180}$ were not. No differences were found in the virus spectra of these 2 strains; thus, the transplantability of strain NCTC 1742 to the C3H mouse served to differentiate them. Strain NCTC 1742 produced tumors, but strain LLC-AS$_{180}$ did not (fig. 1). NCTC 1742 is the "high malignancy" line of the pair of strains developed by Sanford at the National Cancer Institute from an original cloned culture.

The 7 cell strains and the primary rabbit kidney cells, which were refractory to poliovirus and Coxsackie B-5, were further subdivided by differences in their sensitivity to Herpes simplex virus (text-fig. 5). Two mouse strains, LLC-M$_1$ and LLC-M$_3$, were not infected by Herpes simplex virus, but strain LLC-M$_1$ was differentiated from strain LLC-M$_3$ by its susceptibility to B virus. Those strains that did show a CPE from Herpes simplex virus were separated into 2 groups of 3 strains each, of which one was susceptible to vaccinia and the other resistant. The vaccinia-positive group could not be further subdivided by differences in virus sensitivity, but strain LLC-WRC$_{256}$ could be distinguished from the rabbit kidney cells through its transplantability to baby rats. Four of five 4-day-old rats developed tumors after inoculation with strain LLC-WRC$_{256}$, while 6 uninoculated animals in the same litter remained normal. Groups of six 4-day-old baby rats were inoculated with strain LLC-RK$_1$ and with pRK cells, but no tumors developed in these animals over a 41-day period of observation.


Poliovirus –
Coxsackie B-5 –
Herpes simplex
+CPE
–CPE
LLC-MS180, LLC-WRC256, NCTC 2049, LLC-Me1
LLC-RK1, pRK
LLC-M1, LLC-M3
B virus
+CPE
–CPE
LLC-M1
LLC-M3
Vaccinia
+CPE
–CPE
LLC-WRC256, LLC-RK1, pRK
LLC-Me1, LLC-MS180, NCTC 2049
Baby rat transplantability
+Tumor
–Tumor
LLC-WRC256
LLC-RK1, pRK
B virus
+CPE
–CPE
LLC-Me1, LLC-MS180
NCTC 2049
SE polyoma virus
+CPE
–CPE
LLC-Me1
LLC-MS180

TEXT-FIGURE 5.—Further characterization of poliovirus-negative cell strains.

B virus and the SE polyoma virus served to further distinguish between the 3 vaccinia-negative strains. The polyoma virus produced an extensive CPE in strain LLC-Me_1 and hemagglutinin titers up to 1:512, while it failed to cause cell damage in strain LLC-MS_{180}, and the development of hemagglutinins in this strain faded on serial passage.

DISCUSSION

With the few exceptions noted, the 22 cell strains and the 2 types of primary cultures in our laboratory could be identified through differences in their individual spectra of virus sensitivity. It is suggested that similar patterns of reactions with viruses could be established for other cell collections to identify individual strains. The scheme described here worked for one set of cultures, but probably would not be reliable for identification of strains with the same labels maintained in other laboratories, since their past histories and the current methods of propagation might vary considerably. These possible differences in handling strains could lead to alterations in virus sensitivity. An example was given in text-figure 2, where after adaptation to chemically defined medium strain LLC-MK_2 (NCTC 3196) varied with the parent strain LLC-MK_2.

Like the cell strains, different strains of the same virus are likely to vary in their host-cell performances, and this factor must be controlled. It is emphasized that only the specific virus strains with the passage histories indicated in table 2 are applicable to the identification of the cell strains in our collection. Our experience with a different strain of Coxsackie B-5 virus than that listed in the table indicated quite clearly that one must continue to use the same strain and, when possible, the same passage level. After the establishment of the utility of a particular virus seed, reserve samples should be frozen for future use. New pools should be rechecked to confirm that they have the desired properties before they are frozen.

The actual identity of any cell strains used in this study was accurate only insofar as the labels on the strains were accurate at the time the study was begun. Since no two cell strains had identical virus spectra, it was assumed that no single strain was being carried under more than one label.

Though it was not our intent to determine whether certain cell strains were or were not of the animal origin indicated, or to enter into the discussion of cell transformations, certain of these data could be used for such purpose. Pig kidney cells, for example, were susceptible to an old laboratory strain of swine influenza virus, but could not be used for the serial cultivation of 7 other type A and B influenza viruses. The high specificity of S.$V._2$ for rhesus monkey cells proved that this virus is a valuable tool for identification of cells of this species and indicated that the monkey kidney strains were, in fact, monkey in origin. Stulberg *et* *al.* (*3*) recently confirmed this by an immunological technique. Of the remaining cell strains studied, only a few gave unexpected results. The well-known fact that ERK-1 has a virus spectrum more like a human cell than that of a rabbit cell was demonstrated along with its close relation virologically to Det-6. The CH strain of monkey heart cells was resistant to simian viruses, which were readily propagated in other monkey cell strains. The human- and monkey-embryo strains both failed to grow poliovirus at their present passage levels, though both were susceptible to this virus during the early stages of their development. Both were derived from tissues of the torso and consisted mainly of connective-tissue elements. Initially, these 2 strains were 4 to 5 logs less sensitive to poliovirus than were monkey kidney or HeLa cells, and gradually through continued cultivation they became completely resistant. An abrupt change in virus susceptibility or development of a radically different virus spectrum has not been observed in any strains in our collection. Close adherence to a routine of handling cultures, preparation of solutions and equipment, along with the continued use of the same culture medium is believed responsible, in part, for the stability noted.

Since strains LLC-He_1 and LLC-Me_1, both of primate origin, were not susceptible to poliovirus and represented an exception to the rule, an attempt was made to determine if these cells were, in fact, human and monkey cells. Stulberg *et al.* (*3*) recently described a technique for species identification of cell strains by specific staining with fluorescent antibody. Dr. Stulberg kindly offered to examine these 2 strains by this procedure and found that strain LLC-He_1 did stain with antihuman cell fluorescent antibody, but observed also that some cells in the culture stained less intensively with an antimouse cell serum. Strain LLC-Me_1 surprisingly failed to stain, not only with antimonkey cell serum but also with serums prepared against human and mouse cells. Since strain LLC-Me_1 proved so susceptible to polyoma virus, we were concerned about its identity as a monkey cell and reasoned that possibly it was a mouse cell that had been mislabeled. The failure of this strain to stain with any of the 3 serums used by Dr. Stulberg confused the issue, but as he suggested, the embryonic origin of these cells might have influenced the specificity of the test since his antimonkey serum was prepared against adult cells. Before definite significance can be drawn from these studies, the fluorescent-antibody technique must be thoroughly studied with cells of embryonic origin.

A recent experience in our laboratory illustrates the practical application of the virus method of cell-strain identification, and how mistakes in identity can occur. A culture, supposedly of strain LLC-He_1, was removed from the liquid-nitrogen refrigerator, thawed, and planted in culture flasks. After several serial passages the morphological appearance of this strain seemed to vary from that expected and thus raised doubt of its actual identity as LLC-He_1. It was, therefore, subjected to analysis by the virus-susceptibility method described in this report and was identified as HEp-2 instead of LLC-He_1. A recheck of other frozen stocks of strain LLC-He$_1$ indicated that they were not mislabeled. Probably, a tube of HEp-2 had been removed from the refrigerator rather than LLC-He$_1$. In the laboratory, the letters LLC were not carried on the culture flasks, and the 2 was omitted from the HEp-2 strain; thus, the possible error in interpretation between the possibly smudged labels, He$_1$ and HEp, was understandable. If this strain of cells had been shipped out of the laboratory or handled by workers less familiar with its morphological characteristics, another variant cell strain, with a radically different virus spectrum, probably would have been born. If a method for individual strain identification had not been available, this mistake could not have been satisfactorily resolved.

SUMMARY

A method for the identification of 22 cell strains maintained in one collection has been described. This procedure was based primarily on differences in the susceptibility of the various cultures to a selected group of viruses. In two instances the malignant property of 2 strains was employed, and in one case the medium preference of one strain was used to distinguish it from another with the same virus spectrum.

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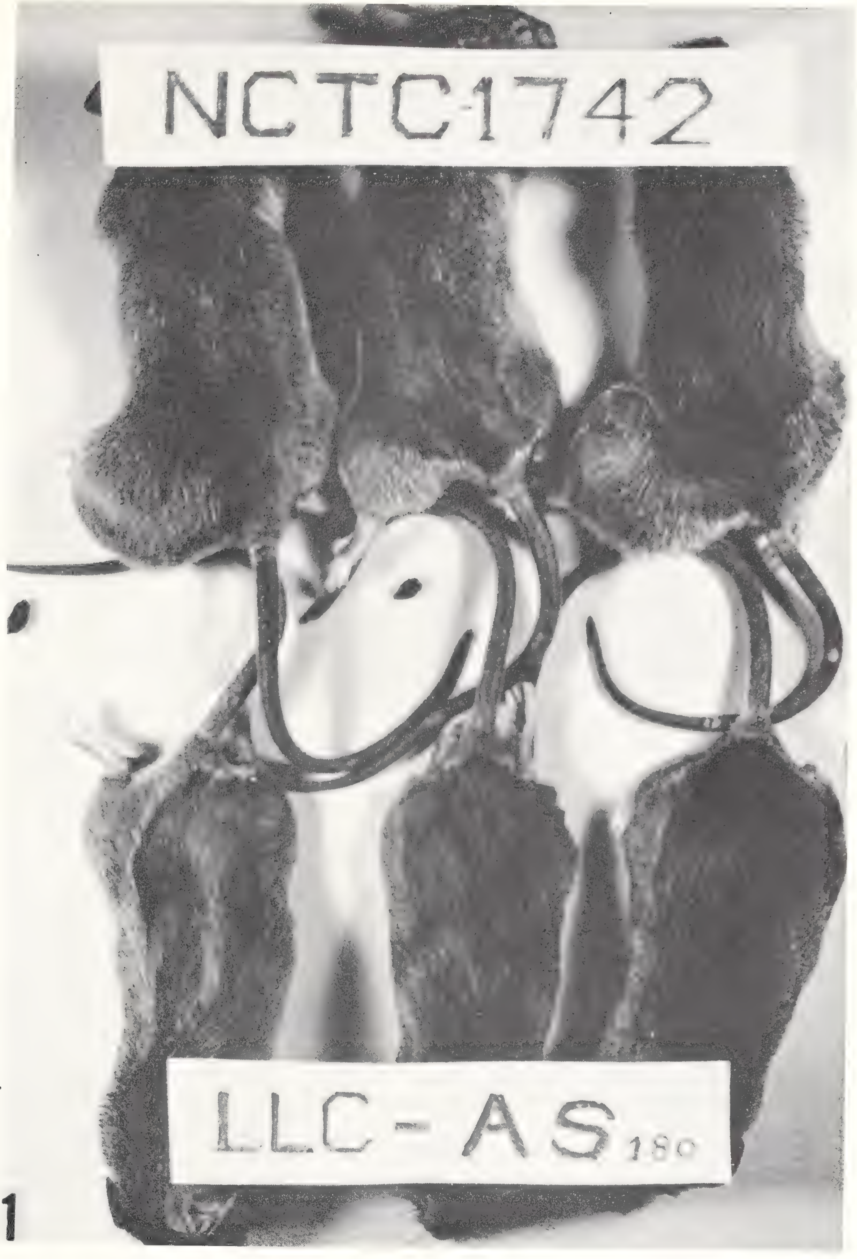
(*2*) Hull, R. N., Minner, J. R., and Smith, J. W.: New viral agents recovered from tissue cultures of monkey kidney cells. I. Origin and properties of cytopathogenic agents S.V.$_{1}$, S.V.$_{2}$, S.V.$_{4}$, S.V.$_{5}$, S.V.$_{6}$, S.V.$_{11}$, S.V.$_{12}$, and S.V.$_{15}$. Am. J. Hyg. 63: 204–215, 1956.

(*3*) Stulberg, C. S., Simpson, W. F., Peterson, W. D., and Berman, L.: Determination of species antigens of cultured cells by immunofluorescence. (Abstract.) Fed. Proc. 20: 150, 1961.

PLATE 15

FIGURE 1.—Transplantability of strain NCTC 1742 to C3H mouse. *Note* lack of tumors in mice with strain LLL=AS_{180} cells.


NCTC 1742
LLC - AS180

1

DISCUSSION

Friedrich Deinhardt, *Presbyterian-St. Luke's Hospital, Chicago, Illinois*

Reading the program of this symposium, I was impressed that more than half of the papers were directly or indirectly concerned with the characterization and stabilization of cultured cell strains. Tissue, or better, cell cultures certainly have given one of the greatest impetuses to virology, and in turn, virologists have broadened the aspects of tissue culture considerably.

The happy marriage of tissue culture and virology became most evident at the Decennial Tissue Culture Association Meeting in Vermont in 1956. I would like to quote one sentence from Dr. Syverton's paper, "The Response of Mammalian cells in Culture to Virus Infection," given at this meeting: "Cell culturists have long been concerned with the nutritional and environmental factors that influence cellular metabolism and morphogenesis; virologists must also comprehend these factors to interpret adequately the response of cells to infection."

The isolation of new cell strains had become almost a social game, especially among virologists hoping to find cell lines which would support the growth of some of their more elusive viruses. Not much success was met on this road, and it came as a shock to many laboratories when the first data on possible contamination of cell cultures with other cells became available. Today, if the isolation of a new cell strain is reported, a great deal of time is spent to prove its identity. It is impossible to discuss here all possible means of cell-line identification, but the long list of different approaches makes it evident that we do not have a single, simple and satisfactory method.

Dr. Hull and his group are to be congratulated for their extensive work on identification of cell lines by virus susceptibility. Whereas immunologic methods so far only elucidate the species origin of a given cell strain and biochemical evaluations are still in their teen-age years, it seems that a detailed analysis with a large battery of viruses really can distinguish between given cell strains even of the same species. However, as Dr. Hull himself pointed out, we should not forget that one and the same cell strain carried in two different laboratories, or even in the same laboratory, in two separate sublines may alter with time. In addition, it is obvious that different strains of the same assay virus may give different results.

Contamination of cell strains with latent viruses has proved another possible pitfall of this assay system, because it is known that inapparently, persistently infected cultures may become more or less resistant to viruses which destroy uninfected cultures in 24 hours. Such a system employing MCN or strain L cells and myxoviruses, as the latent and vesicular stomatitis (VSV) as the virulent virus, has been studied by the Drs. Henle and myself for several years. It has been proved that in the MCN-myxovirus system enough interferon is produced to render such persistently infected cultures almost completely resistant to VSV and to a lesser extent to other cytopathic viruses. A characterization of such a cell culture by virus spectrum obviously would give erroneous results.

In summary, we can say that we need a number of different evaluations, such as morphology, chromosome and biochemical analysis, immunologic tests, and evaluation of virus spectrum, to determine the identity of a given cell strain. We have a long way to go to find *more precise* criteria for the origin of cultured cells, and we are still far from able to call our laboratory cell strains "*stable*." In other words, we are faced with the problem of identifying accurately a biological system which is constantly changing in our hands, and we can apply John Ward's words on identity: "Resemblance itself may be fatal to identification, when the law of being is change."

Assessment of Potential Malignancy of Cultured Cells: Further Observations on the Differentiation of "Normal" and "Neoplastic" Cells Maintained *In Vitro* by Heterotransplantation in Syrian Hamsters[1,2]

G. E. FOLEY, A. H. HANDLER, R. A. ADAMS, *and* J. M. CRAIG,[3] *Laboratories of Microbiology and Tumor Transplantation, The Children's Cancer Research Foundation, and Department of Pathology, Harvard Medical School, at The Children's Hospital Medical Center, Boston, Massachusetts*

MAMMALIAN CELLS in culture, whether derived from normal or neoplastic sources, are not only morphologically (*1–8*) and, in some respects, biochemically similar (*9*, *10*), but also have responded to a variety of growth inhibitors in essentially the same manner (*11–13*). Nevertheless, after it was established that cell lines of neoplastic origin could be transplanted from culture and titrated in the hamster cheek pouch (*14*), a preliminary study indicated that cells of neoplastic and normal origin could indeed be distinguished from one another in that only cells of neoplastic origin grew progressively below the critical inoculum of 1.0×10^4 cells in unconditioned hamsters, or 1.0×10^3 cells in cortisone-conditioned hamsters (*15*, *16*). These results were not unexpected, in view of the original observation by Handler *et al.* (*17–19*) that the cheek pouch of the Syrian hamster (*Mesocricetus auratus*) accepts heterotransplants of neoplastic tissue, and the subsequent observation that the Syrian hamster exhibits the additional interesting attribute of ultimately rejecting adult normal (but not necessarily embryonal) heterologous tissue (*17*, *19*).

The present report describes an extended exploration of this phenomenon to include 63 cell lines, of which 35 were isolated in these laboratories.

METHODS

Cell lines.—The 63 established mammalian cell lines, assembled for these studies during more than 4 years, include 23 derived from neoplastic, 28 from normal[4] adult, and 9 from normal embryonal tissue. The remain-

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[1] Presented at the Syverton Memorial Symposium on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

[2] These studies were supported in part by research grants C-2230 (C), C-2782, and CY-3335, from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] *Present address:* Department of Pathology, Boston Lying-In Hospital, Boston, Mass.

[4] The term "normal," as used in this context, connotes the absence of known neoplastic, but not necessarily other disease.

ing 3 were isolated from benign tumors. Thirty-five of these, the *CCRF*[5] *cell lines*, are listed in table 1, together with their tissue of origin and date of isolation. Details of their isolation in Eagle's medium (*9*) or modifications thereof, directly from biopsy and surgical specimens, are provided elsewhere (*20*). The remaining 28 obtained from other sources are similarly listed in table 2.

TABLE 1.—Source of CCRF cell lines studied in Syrian hamsters*

Cell line, CCRF No.	Tissue of origin†	Date isolated (month/year)
	Normal human	
200	Amnion at term (*13, 15, 16*)	8/56
201	Endometrium, therapeutic abortion (*15, 16*)	5/56
202	Biopsy, lung, chronic pneumonitis (*15, 16*)	6/56
203	Biopsy, epitrochlear lymph node, infectious granuloma, etiology?	8/56
204	Biopsy, thymus (*13, 15, 16*)	6/56
205	Biopsy, cervical lymph node	4/57
206	Biopsy, lung	6/57
207	Biopsy, inguinal lymph node	6/57
209	Nasal polyp, cystic fibrosis	7/58
210	Nasal polyp	12/56
211	Biopsy, femoral ulcer, etiology?	2/57
212	Nasal polyp, cystic fibrosis	4/57
213	Nasal polyp, adult	4/57
214	Pleural effusion, nonspecific etiology	1/58
215	Fetal epidermis, therapeutic abortion	10/56
	Normal animal	
208	Lung, Chinese hamster	2/57
216	Spleen, normal DBA/2 mouse	1/58
217	Testes, normal DBA/2 mouse	1/58
218	Embryonal liver, DBA/2 mouse	12/57
219	Embryonal skin, CFW mouse	11/57
	Malignant human	
6	Surgical, Wilms' tumor (*15, 16*)	7/56
100	Surgical, lung, lymphoma	6/57
104	Biopsy, melanotic melanoma, adult	1/57
105	Biopsy, melanotic melanoma, adult	4/56
106	Biopsy, lymphoma, from primary tumor isolated in Syrian hamster‡	10/57
107	Biopsy, melanotic melanoma, adult	9/57
108	Biopsy, laryngeal carcinoma, adult	12/56
	Benign human	
102	Surgical, astrocytoma	3/57
103	Surgical, ependymoma	3/57
	Malignant animal	
91	Cloudman melanoma, S91 in DBA/1 mice (*21*)	4/57
101	Ascitic fluid, Ehrlich ascites in CFW mice (*22, 23*)	2/57
180	Sarcoma 180 in CFW mice (*15, 16, 24*)	6/55
1210	Ascitic fluid, L1210 lymphoid leukemia of DBA/2 mice	5/58
1534	Serially passed tumor in Syrian hamsters, P1534 lymphatic leukemia of DBA/2 mice‡ (*14, 25*)	3/57
109	DBRB mammary adenocarcinoma of DBA/1 mice	11/58

**Cf.* (*20*) for details of isolation.

†Human specimens from pediatric age groups; animal specimens from adults unless otherwise specified.

‡Isolated from biopsy specimen in Syrian hamsters by Dr. A. H. Handler.

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[5] Children's Cancer Research Foundation

Table 2.—Source of other cell lines studied in Syrian hamsters

Cell line	Tissue of origin*	Date isolated (month/year)
	Normal human	
C	Conjunctiva† (*26*)	2/54
407	Embryonal intestine† (*27*)	1/55
D-189	Biopsy, foreskin‡ (*28*)	9/53
L	Biopsy, liver† (*26*)	4/54
407	Embryonal liver† (*27*)	1/55
K	Biopsy, kidney§ (*26*)	5/54
Heart	Biopsy, commissurotomy‡ (*29*)	2/56
MAF-83	Embryonal skin and muscle‡ (*30*)	11/57
LLC-HE·1	Embryo‖ (*31*)	8/54
Detroit-52	Sternal bone marrow, diabetes mellitus¶ (*3*)	8/55
Detroit-98	Sternal bone marrow, refractive error¶ (*3*)	11/55
Detroit-173B	Buffy coat, peripheral blood¶ (*4*)	5/56
Detroit-196Ep-L	Biopsy, skin, urticaria pigmentosa¶ (*32*)	8/56
	Normal animal	
L-929	Biopsy, connective tissue, C3H mouse† (*33*)	10/40
LLC-M-1	Hypertrophic lymph node, leg of white mouse of unknown origin‖ (*31, 34*)	10/51
Bovine kidney	Embryonal‡ (*30*)	8/57
Bovine skin & muscle	Embryonal‡ (*30*)	8/57
	Malignant human	
HeLa	Biopsy, cervical carcinoma† (*35*)	/51
KB	Biopsy, epidermoid carcinoma of floor of mouth† (*36*)	12/54
J-111	Buffy coat, peripheral blood, monocytic leukemia† (*37*)	8/54
Detroit-6	Sternal marrow, bronchiogenic carcinoma¶ (*3, 4, 38*)	9/54
Detroit-30a	Ascitic fluid, mammary carcinoma¶ (*3, 4*)	1/55
Detroit-32	Sternal marrow, carcinomatosis¶ (*3, 4*)	2/55
Detroit-34	Sternal marrow, metastatic carcinoma of bone¶ (*3, 4*)	4/55
Detroit-56A	Ascitic fluid, carcinomatosis¶ (*3, 4*)	7/55
Detroit-116P	Pleural fluid, lymphosarcoma¶ (*3, 4*)	9/55
	Benign human	
Detroit-501	Hamartoma, 5-day-old infant¶ (*4*)	1/56
	Malignant animal	
Ca-755	Biopsy, Ca-755 in DBF/1 mice‡ (*39*)	10/56

*From adults, unless otherwise specified.

†The strain used in these studies was obtained through the courtesy of: Dr. Harry Eagle, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

‡Dr. A. J. Girardi, Microbiological Associates, Inc., Bethesda, Md.

§Dr. R. M. McAllister, Camden Municipal Hospital, Camden, N.J.

‖Dr. R. N. Hull, Lilly Research Laboratories, Indianapolis, Ind.

¶Dr. C. S. Stulberg, The Child Research Center of Michigan, Detroit, Mich.

Serial propagation and maintenance.—To minimize the risk of loss due to bacterial or fungus contamination, all cell lines were maintained in duplicate, by different personnel, as completely separate, viable, monolayer stock cultures in 2.5 to 3.0 ml of Eagle's medium in culture flasks[6] with a surface of approximately 15 cm^2, which were closed with tight-fitting, siliconized, rubber stoppers.[7] The basal medium was supplemented with 10 percent whole, pooled human serum[8] for cell lines of human origin, while 5 or 10 percent whole calf[8] or horse serum[9] was used for cell lines of animal origin (*20*). All cultures were incubated stationary at 37° C and subcultured at approximately weekly intervals.

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[6] Modified T-15 flasks (Earle, W. T., and Highhouse, F.: J. Nat. Cancer Inst. 14: 841–850, 1954).

[7] The West Company, Phoenixville, Penna.

Preparation of cells for implantation in cheek pouch.—It must be emphasized that the reproducibility of the results obtained with a given cell line in hamsters is dependent in large part on the physiologic and metabolic condition of the cells in the inoculum. Cells that are growing poorly *in vitro* will not grow in the hamster cheek pouch, irrespective of the origin of the cell line, and suspensions prepared in inappropriate diluents, *e.g.*, "physiological" saline, even from suitable, actively growing cultures, frequently fail to grow in the cheek pouch.

Subjective criteria for vigorous metabolic activity in the mammalian cell culture systems used herein may appear satisfactory, but even after considerable experience, often prove misleading when subjective opinion is compared with objective measurement. The criterion used to adjudge vigorous metabolic activity in the present studies was the same as that used previously in these laboratories (*11–13*): a fivefold to tenfold increase in the total protein content of the culture within 6 to 8 days, as determined by the method described by Oyama and Eagle (*40*). Cell lines that are not growing at least at this rate in culture have, in general, been unsatisfactory for hamster experiments.

To assure that cells were in the log-phase of growth, all inocula were prepared from 5-day-old transplants of stock cultures. All operations were done in complete culture media to preserve, as much as possible, the physiologic and morphologic integrity of the cells. The seed cultures were dispersed with 0.25 to 0.125 percent trypsin,[10] as described previously (*14, 15, 20*), washed, and subcultured to 1 liter Blake bottles (surface area *ca.* 150 cm^2) in 60 to 70 ml of media supplemented with appropriate whole serum. The exhausted medium was renewed daily, and after 5 days' incubation at 37° C, the resulting layer of cells was again dispersed with trypsin, sedimented at 50 to 70 $\times$ *g*, and washed 3 times with complete media. The washed cells were redispersed with gentle agitation in an appropriate volume of complete medium and enumerated by hemocytometer counts. The suspension of cells was then diluted with complete medium on the basis of the mean of 3 or more hemocytometer counts in such a way that the desired inoculum was contained in 0.1 ml of the final suspension. Inocula prepared in this manner contain 90 to 95 percent viable cells, as adjudged by the usual trypan blue technique (*41*).

The preparation of inocula was scheduled so that final implantation into the cheek pouch was accomplished within 1 to 2 hours of elapsed time.

Implantation of cells in cheek pouch.—The cheek pouch of the Syrian hamster may be everted under light anesthesia and thus becomes readily accessible for direct manipulation and observation (fig. 1). The pouch is a well-vascularized, transparent membrane consisting of 2 thin layers of epithelium separated by a thin layer of connective tissue (*42*) (fig. 2).

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[8] Microbiological Associates, Inc., Bethesda, Md.

[9] Supplied through courtesy of Antitoxin and Vaccine Laboratories, Massachusetts Department of Public Health, Jamaica Plain, Mass.

[10] Difco "1:250," Difco Laboratories, Inc., Detroit, Mich.

Syrian hamsters, 60 to 80 g in weight, were prepared under light Nembutal[11] anesthesia as described previously (*14*, *15*), the cheek pouch everted and pinned to a cork board designed for this purpose. The vials containing the appropriately diluted cells were agitated sufficiently to assure uniform suspension and 0.1 ml was drawn into a sterile 1.0 ml tuberculin syringe through a 24-gauge needle. It is important that each dose be handled in the syringe separately, otherwise unavoidable sedimentation of the cells results in considerable error in the number of cells contained in successive inocula. Separate syringes and needles were used for each dilution of cells. The 0.1 ml of culture medium containing the desired cell inoculum was implanted into the upper layer of pouch epithelium as an intradermal or intracutaneous bleb, as illustrated in figures 1, 3, and 4. Both cheek pouches of each of 6 hamsters were implanted with each dilution of each cell line. Three hamsters in each group were "conditioned" with cortisone acetate[12] in subcutaneous doses of 2 to 3 mg, administered simultaneously with implantation, and twice weekly post implantation (*14*, *15*, *43*, *44*).

Method of evaluation.—The cheek pouches of all hamsters were observed twice weekly under light Nembutal anesthesia for at least 60 days. The development of a nodule that became vascularized and grew progressively was considered as evidence of growth. Histological sections were prepared from all cheek pouches in which such growth occurred. Since at least 2 experiments were done with the majority of cell lines, the data considered herein were derived from observations on *circa* 7,000 hamsters. Personnel responsible for the gross evaluation of the cheek pouches were unaware of the origin of a given cell line, and similarly, histological examination of the excised cheek pouches was made without prior knowledge of the nature of the tissue from which the cell line was derived.

Although there are distinct differences in the behavior of cell lines derived from neoplastic and non-neoplastic sources, even when large inocula (1.0×10^6 or 1.0×10^5 cells) are implanted in the cheek pouch, the growth of small inocula (1.0×10^4 cells in unconditioned, and 1.0×10^3 cells in cortisone-conditioned hamsters) is a more sensitive differential criterion. The actual comparison made with these cell lines, therefore, using these small inocula, was on the basis of their ability to produce progressively growing, vascularized nodules in at least 2 of the 6 pouches in each group of 3 hamsters implanted with inocula of neoplastic origin, and conversely, the inability of cell lines of non-neoplastic origin to survive and grow in the cheek pouch following implantation of similar inocula. These differences in behavior in the hamster cheek pouch are illustrated by the experiments summarized in figures 5 through 20, wherein the cheek pouches inoculated with a cell line of neoplastic origin (J–111, *cf.* table 2) and a cell line of normal origin (Detroit–173B, *cf.* table 2) were photographed successively at 7-day intervals.

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[11] Veterinary Nembutal, sodium, 60 mg/ml; Abbott Laboratories, North Chicago, Ill. Administered intraperitoneally, 0.15 ml per 100 g body weight.

[12] Cortone acetate, 25 mg per ml in saline suspension; Sharpe and Dohme Division, Merck and Co., Inc., Philadelphia, Penna.

The tumors resulting from the implantation of 1.0×10^5, 1.0×10^4, and 1.0×10^3 cells of neoplastic origin are illustrated in figures 5 through 10. Such nodules characteristically increase progressively in size, become vascularized, and frequently fill the pouch, presenting the gross appearance of a typical cheek pouch tumor (figs. 11 and 12). After varying lengths of time, the tumors so produced become necrotic and are reabsorbed, especially in unconditioned hamsters, and hamsters harboring necrotic tumors invariably die of intercurrent infection. Tumors excised prior to extensive necrosis are transplantable, and may be carried by serial transplant in the cheek pouch. This sequence of events is characteristic in all pouches successfully implanted with cell lines of neoplastic origin (*cf.* tables 3 and 6). The incidence of "takes" varied with different cell lines, usually 4 to 6, but occasionally as few as 2 or 3 tumors were obtained in the 6 pouches of each of the groups of 3 hamsters.

In contrast to these observations, the nodules resulting from the implantation of 1.0×10^6 or 1.0×10^5 cells of normal origin do not grow progressively in the cheek pouch, but rather necrose rapidly, and do not persist for more than a few days. As illustrated in figures 13 through 20, the nodules produced by the implantation of 1.0×10^5 cells of Detroit-173B had disappeared completely, even in the pouches of cortisone-conditioned hamsters by the 14th postimplantation day. Even if excised prior to necrosis and reabsorption, such nodules cannot be transplanted to other hamsters. This sequence of events (with few exceptions, *cf.* table 4) is characteristic of the pouches implanted with 1.0×10^6 or 1.0×10^5 cells of nonmalignant origin. Smaller inocula (1.0×10^4–1.0×10^1 cells) usually do not even produce these transient "nodules," the "bleb" produced by the implant having disappeared between 24 and 48 hours.

RESULTS

Cell Lines Derived From Malignant Tissue

The behavior of each of 17 cell lines (the Detroit cell lines are considered separately) isolated from neoplastic tissue in the hamster cheek pouch is presented in table 3. The first 11 cell lines listed in table 3 differ from those cell lines derived from normal adult tissue in that each grew consistently and repeatedly after implantation of 1.0×10^4 cells in *unconditioned* as well as in cortisone acetate-conditioned hamsters. Ten of these cell lines also grew from 1.0×10^3 cells, and 5 (KB, HeLa, CCRF-91, J-111, and CCRF-1210) grew from 1.0×10^2 cells in *unconditioned* hamsters, while 6 cell lines produced tumors in cortisone-conditioned hamsters after implantation of about 10 cells (table 3).

TABLE 3.—Behavior of cell lines derived from malignant sources in the Syrian hamster cheek pouch

	Growth after implantation* of cells at:											
	1.0×10^6		1.0×10^5		1.0×10^4		1.0×10^3		1.0×10^2		10	
Cell line	C	NC	C	NC	C	NC	C	NC	C	NC	C	NC
Human												
CCRF-6	+	+	+	+	+	+	+	+	0	0	0	0
KB	+	+	+	+	+	+	+	+	+	+	+	+
HeLa	+	+	+	+	+	+	+	+	+	+	+	+
J-111	+	+	+	+	+	+	+	+	+	+	0	0
CCRF-106	+	+	+	+	+	+	+	0				
Animal												
CCRF-91	+	+	+	+	+	+	+	+	+	+	+	0
CCRF-101	+	+	+	+	+	+	+	+	+	0	+	0
CCRF-180	+	+	+	+	+	+	+	0	+	0	+	0
CCRF-1210	+	+	+	+	+	+	+	+	+	+	+	0
CCRF-1534	+	+	+	+	+	+	+	+	0	0	0	0
Ca-755	+	+	+	+	+	+	+	+	0	0	0	0
CCRF-109	+	+	+	+	+	+	+	+				
Human												
CCRF-107	+	+	+	0	0	0						
CCRF-100	+	+	0	0	0	0	0	0	0	0	0	0
CCRF-105	+	+	0	0	0	0						
CCRF-104	+	0	0	0	0	0						
CCRF-108	0	0	0	0	0	0						

* "NC" = untreated; "C" = cortisone-conditioned hamsters; *cf.* text.

The 5 remaining cell lines in this group are included in table 3, since they were derived from patients with neoplastic disease, though they exhibit atypical behavior in the hamster cheek pouch. However, these cell lines are not considered contradictions to the general concept of the behavior of neoplastic cells in the hamster cheek pouch. Histological evaluation indicates quite clearly that it is improbable that these particular cell lines are of neoplastic origin, since in each instance the original biopsy specimen contained relatively few viable tumor cells. For example, the biopsy specimen from which CCRF-107 was isolated consisted largely of degenerating and acute inflammatory cells, with but a small focus of viable tumor cells (fig. 21). More than one half the total area of the biopsy specimen from which CCRF-108 was isolated consisted of normal connective tissue (fig. 22). The few identifiable neoplastic cells were grouped along one edge, and in only two areas of the specimen was there any evidence of invasion. Similarly, the biopsy specimen from which CCRF-100 was isolated was heavily infiltrated with inflammatory cells, eosinophils, lymphocytes, mononuclear and plasma cells, and contained many areas of necrosis, abscess formation, and scarring. Indeed, aside from the occasional Sternberg-Reed cell and an occasional blast form of the mononuclear series, it was difficult to designate either single cells or areas of tissue as neoplastic (fig. 23).

In the 3 cell lines, CCRF-104, -105, and -107 isolated from human melanomas, the rare pigmented cells in primary cultures disappeared during the first few passages *in vitro*, resulting in what appeared to be "pure cultures" of "fibroblast-like" cells. Similarly, the other 2 cell lines in this group (CCRF-100 and -108) also are of "fibroblast-like" morphology. The nidus resulting from the implantation of 1.0×10^6 cells of each of these 5 cell lines into the hamster cheek pouch consisted of occasional surviving cells scattered in a dense accumulation of inflammatory cells and cellular debris which had elicited a marked stromal and inflammatory response on the part of the host, as illustrated in figure 24, and as has been described previously with cell lines of normal origin (*16*).

The direct implantation of these biopsy specimens into the cheek pouches of cortisone-conditioned hamsters also failed to result in the isolation of a tumor line in all but one instance. The original biopsy specimen of the melanoma from which CCRF-107 was derived grew in the hamster cheek pouch as small, scattered foci of melanomatous cells that could be serially transplanted in the cheek pouch only after careful selection and dissection of the small melanomatous foci for subtransplants.

Cell Lines Derived From Normal Tissue

The behavior of each of 37 cell lines isolated from normal adult or embryonal tissue in the hamster cheek pouch is presented in table 4. Of the 28 cell lines derived from adult normal tissue, only 3 grew in the cheek pouches of cortisone-conditioned hamsters after implantation of 1.0×10^4 cells. Two of these cell lines (Detroit-52 and K, *cf.* table 4) grew consistently from this inoculum, while the third (L-929, *cf.* table 4) was variable and failed to grow at 1.0×10^4 cells in most experiments done with this strain. However, with the exception of Detroit-52 (table 4), none of these 28 cell lines grew in the cheek pouches of *unconditioned* hamsters when the inoculum contained 1.0×10^4, or fewer, cells.

The Detroit-52 cell line, isolated from the sternal bone marrow of a female patient with diabetes mellitus (*3*) (table 2) has grown consistently in the cheek pouches of unconditioned hamsters from inocula of 1.0×10^4 cells in all experiments over the course of 3 years with several different subcultures derived from the original parent stock.[13] Insofar as can be determined, this patient presents no evidence of neoplastic disease,[14] and there is no explanation for the apparently aberrant behavior (if Detroit-52 is indeed "normal") of this cell line.

Other cell lines of normal origin differ somewhat from each other in their capacity to survive and grow in the cheek pouch (table 4), some growing at 1.0×10^5 cells in either unconditioned or cortisone-conditioned hamsters, *e.g.*, D-189, some growing at 1.0×10^5 cells only in cortisone-conditioned hamsters, *e.g.*, CCRF-200, and others failing to grow at

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[13] We wish to thank Dr. C. S. Stulberg, The Child Research Center of Michigan, Detroit, Mich., for providing these subcultures.

[14] Personal communications, Dr. Lawrence Berman, Department of Pathology, College of Medicine, Wayne State University, Detroit, Mich.

1.0 × 10^5, *e.g.*, CCRF-201, or even at 1.0 × 10^6 cells, *e.g.*, CCRF-202, in either unconditioned or cortisone-conditioned hamsters. Despite such differences, however, all cell lines except Detroit-52 are similar in that

TABLE 4.—Behavior of cell lines derived from normal sources in the Syrian hamster cheek pouch

	Growth after implantation* of cells at:											
	1.0×10^6		1.0×10^5		1.0×10^4		1.0×10^3		1.0×10^2		10	
Cell line	C	NC	C	NC	C	NC	C	NC	C	NC	C	NC
Adult human												
Detroit-52	+	+	+	+	+	+†	0	0	0	0	0	0
K	+	+	+	+	+	0	0	0	0	0	0	0
D-189	+	+	+	+	0	0	0	0	0	0	0	0
CCRF-203	+	+	+	+	0	0	0	0	0	0	0	0
CCRF-204	+	+	+	+	0	0	0	0	0	0	0	0
CCRF-205	+	+	+	+	0	0	0	0	0	0	0	0
CCRF-213	+	+	+	+	0	0	0	0	0	0	0	0
Detroit-98	+	+	+	+	0‡	0	0	0	0	0	0	0
Detroit-173B	+	+	+	+	0	0	0	0	0	0	0	0
Detroit-196	+	+	+	+	0	0	0	0	0	0	0	0
C	+	+	+	+	0	0	0	0				
CCRF-200	+	+	+	0	0	0	0	0				
Heart	+	+	+	0	0	0	0	0				
CCRF-210	+	+	+	0	0	0						
L	+	+	0	0	0	0	0	0				
CCRF-201	+	+	0	0	0	0						
CCRF-206	+	+	0	0	0	0						
CCRF-207	+	+	0	0	0	0						
CCRF-211	+	+	0	0	0	0						
CCRF-212	+	+	0	0	0	0						
CCRF-202	0	0	0	0	0	0						
CCRF-209	0	0	0	0	0	0						
CCRF-214	0	0	0	0	0	0						
Adult animal												
L-929	+	+	+/0§	+/0§	+/0§	0	0	0	0	0	0	0
CCRF-208	+	+	+	+	0	0	0	0				
LLC-M-1	+	+	+	+	0	0	0	0				
CCRF-216	+	+	0	0	0	0	0	0				
CCRF-217	+	+	0	0	0	0	0	0				
Embryonic human												
Intestine-407	+	+	+	+	+	+	+	+	0	0	0	0
LLC-HE-1	+	+	+	+	+	+	+	0	+	0	0	0
Liver-407	+	+	+	0	0	0	0	0	0	0	0	0
CCRF-215	+	+	0	0	0	0						
MAF-83	+	+	0	0	0	0						
Embryonic animal												
CCRF-219	+	+	+	+	+	+	0	0	0	0		
CCRF-218	+	+	0	0	0	0						
Bovine kidney	0	0	0	0	0	0						
Bovine skin and muscle	0	0	0	0	0	0						

*"NC" = untreated; "C" = cortisone-conditioned hamsters; *cf.* text.

†Evidence of growth obtained in 7 of 18 pouches in untreated hamsters, as compared to 6 of 18 pouches in untreated hamsters at 1.0 × 10^5 cells.

‡ Evidence of growth obtained in 1 of 24 pouches in cortisone-conditioned hamsters, as compared to 12 of 24 pouches in cortisone-conditioned hamsters at 1.0 × 10^5 cells.

§ Evidence of growth obtained in 5 of 18 and 3 of 18 pouches in untreated and cortisone-conditioned hamsters, respectively, at 1.0 × 10^5 cells, and in 3 of 18 pouches in cortisone-conditioned hamsters at 1.0 × 10^4 cells.

all failed to grow from inocula of 1.0×10^4, or fewer, cells in *unconditioned* hamsters.

Cell Lines Derived From Normal Embryonal Tissue

These cell lines varied considerably in their capacity to survive and grow in the hamster cheek pouch (table 4). Some failed to grow completely, *e.g.*, bovine skin and muscle, others grew only from inocula of 1.0×10^6 cells, *e.g.*, CCRF-218, while 3 cell lines, Intestine 407, LLC-HE-1, and CCRF-219, grew from inocula of 1.0×10^4 cells, and 2 of these, Intestine 407 and LLC-HE-1, grew from inocula containing less than 1.0×10^4 cells. With respect to their ability to survive and grow in the hamster cheek pouch, it appears that certain embryonal cell lines resemble those derived from adult normal tissue, whereas others exhibit considerably more "growth potential" in the hamster cheek pouch.

Cell Lines Derived From Benign Tissue

The behavior of each of 3 cell lines isolated from benign (human) tumors in the hamster cheek pouch is presented in table 5. All 3 cell lines resemble those derived from adult normal tissue in that each failed to grow from 1.0×10^4 cells in either unconditioned or cortisone-conditioned hamsters.

TABLE 5.—Behavior of cell lines derived from benign tumors in the Syrian hamster cheek pouch

	Growth after implantation* of cells at:											
	1.0×10^6		1.0×10^5		1.0×10^4		1.0×10^4		1.0×10^2		1.0×10	
Cell Line	C	NC	C	NC	C	NC	C	NC	C	NC	C	NC
Detroit-501	+	+	+	+	0	0	0	0	0	0	0	0
CCRF-102	+	+	+	0	0	0	0	0				
CCRF-103	0	0	0	0	0	0	0	0				

* "N" = untreated; "C" = cortisone-conditioned hamsters; *cf.* text.

Detroit Cell Lines

The behavior of each of 11 of the Detroit cell lines in the hamster cheek pouch is presented in table 6. The cell lines in this group, which were derived from patients without evidence of neoplastic disease (Detroit-52, -98, -173B, -196 Ep-L, and -501) (*3*, *4*, *32*) already have been considered (*cf.* tables 2, 4, and 5). The remaining 6 Detroit cell lines were derived from the sternal marrow or the ascitic or pleural fluid of patients with neoplastic disease (*3*, *4*, *38*). In each instance, there was histological as well as other evidence for neoplasia, though the histological evidence was not always obtained from the same specimen as that from which the cell line was derived (*3*).

As indicated in table 6, the cell lines derived from patients with no evidence of neoplastic disease failed to survive and grow in the cheek pouches of cortisone-conditioned or unconditioned hamsters when the inoculum contained 1.0 × 10^4, or fewer, cells, with the one exception (Detroit-52) previously discussed. Four of the cell lines derived from patients with evidence of neoplastic disease produced tumors in the hamster cheek pouch at 1.0 × 10^4 cells. The remaining 2 cell lines (Detroit-6 and -116P) that failed to exhibit this growth potential in the hamster cheek pouch were derived from specimens which undoubtedly contained non-neoplastic cells. Although the Detroit cell lines cannot be distinguished from one another on the basis of cellular morphology (*4*, *45*), it is of interest that Detroit-116P exhibits subtle differences in its pattern of growth *in vitro* which may distinguish it from the cell lines isolated from patients with no evidence of malignant disease (*3*).

TABLE 6.—Behavior of Detroit cell lines* in the Syrian hamster cheek pouch

Detroit cell line	Tissue of origin	Diagnosis of donor patient	Growth in untreated and cortisone-conditioned hamsters after implantation of cells at:		
			1.0 × 10^6	1.0 × 10^5	1.0 × 10^4
6	Sternal marrow	Bronchiogenic carcinoma	+	+	0
30a	Ascitic fluid	Mammary carcinoma	+	+	+
32	Sternal marrow	Carcinomatosis	+	+	+
34	Sternal marrow	Metastatic carcinoma, bone	+	+	+
56A	Ascitic fluid	Carcinomatosis	+	+	+
116P	Pleural fluid	Lymphosarcoma	+	+	0
501	Biopsy	Hamartoma	+	+	0
52	Sternal marrow	Diabetes mellitus	+	+	+†
98	Sternal marrow	Refractive error	+	+	0‡
173B	Peripheral blood	Normal adult	+	+	0
196Ep-L	Biopsy, skin	Urticaria pigmentosa	+	+	0

**Cf.* (*3*, *4*, *32*, *38*).

†No evidence of neoplastic disease in donor; *cf.* text.

‡Evidence of growth in 1 of 24 pouches in cortisone-conditioned hamsters.

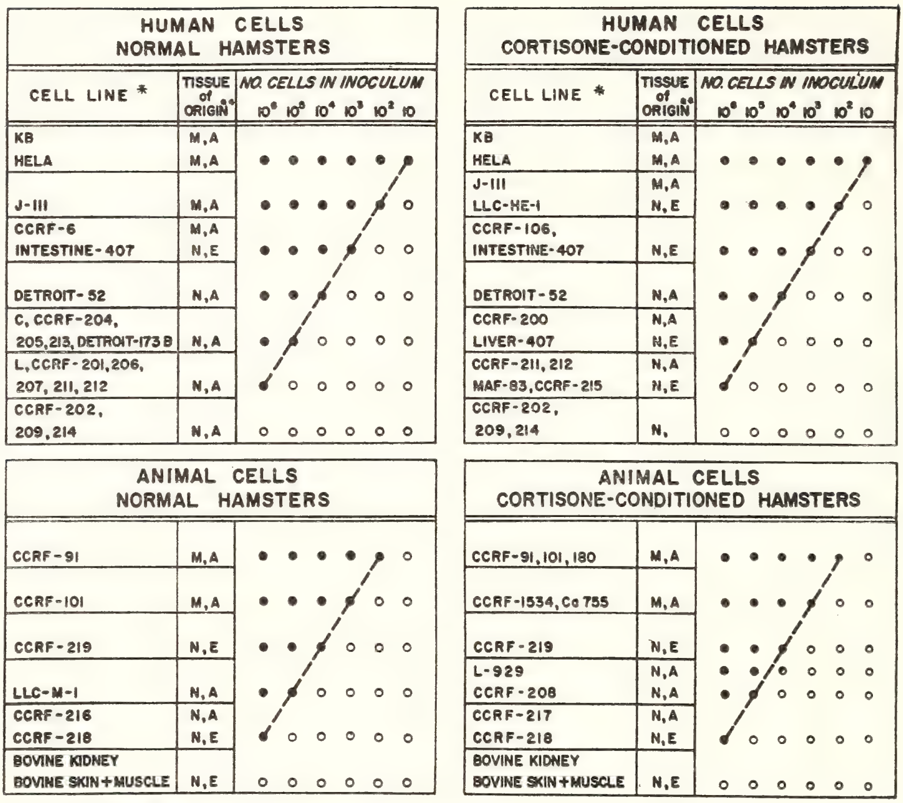
DISCUSSION

The progressive growth of homotransplanted biopsy specimens of rodent tissues is the classical diagnostic criterion of experimental neoplasia, though failure of such specimens to grow does not necessarily indicate that they are non-neoplastic. The application of this criterion to the erratic behavior of homotransplanted rodent cell cultures derived from non-neoplastic sources has raised divergent views as to whether such cell lines are "normal" or "neoplastic" (*28, 46–52*), and has supported the concept that normal human cells are "transformed" to neoplastic cells consequent to cultivation *in vitro* (*5, 28, 53*).

Extensive homotransplantation studies in man are, of course, impossible, though a few studies have been reported with human biopsy specimens (*54*) and cultured cell lines (*53, 55–58*). Consequently, the transplantability of biopsy specimens or cultivated cell lines of human origin must be investigated largely in heterologous hosts. Heterotransplantation studies with cortisone-treated or X-irradiated mice, rats, and Syrian hamsters (*17, 19, 22, 25, 43, 44, 59–76*) are well known, as are the studies of Greene (*77–80*) on the capacity of the anterior chamber of the guinea pig to distinguish between biopsy specimens of neoplastic and normal tissue. The hamster experiments reported herein differ from other heterotransplantation studies in that a combination of quantitative methods and a unique host was used: Cell inocula were titrated and a comparison was made between cell lines from differences in the behavior of small inocula in the cheek pouch of the hamster. A similar quantitative method was used by Andervont and Shimkin (*81*) to demonstrate differences in the growth and survival of neoplastic and normal tissue in isologous and homologous host mice. Mammalian cell cultures also have been titrated in conditioned rats, but despite encouraging preliminary results (*82*), McAllister and Coriell (*83*) concluded recently that the conditioned rat is not sufficiently sensitive to the implantation of heterologous cells to permit precise differential titration. The demonstration of titrable differences among the cell lines used in this study is no doubt due in part to the distinctive morphologic properties of the hamster, namely, the accessibility, translucency, and thinness of the hamster cheek pouch, which permit the repeated accurate observation of the behavior of small inocula. Of even more importance is the unique response of the Syrian hamster to heterografted tissue, as evidenced by the studies of Handler (*25*) *et al.* (*76*), who have shown that certain mouse lymphomas are serially heterotransplantable in normal adult hamsters, with retention of genetic identity of the grafts, as demonstrated by their strain-specific retransplantability in mice. Further evidence is afforded by the well-established observation that a variety of human neoplasms are transplantable in hamsters with a minimum of host-conditioning in the form of subcutaneous injections of cortisone-acetate (*74, 75*). Billingham *et al.* (*84*) have shown that fragments of skin in the hamster cheek pouch escape the homograft reaction and propose that the hamster cheek pouch enjoys a "privileged status" immunologically. These morphologic and physiologic characteristics of the hamster permit the extended study not only of cell lines of neoplastic origin, but those of normal origin as well, prior to rejection by the host. Thus, in the present experimental system, it is possible to distinguish between cells of normal and neoplastic origin at inocula of 1.0×10^4 cells, an *ipso facto* demonstration that all cell lines maintained *in vitro* are not "malignant." Despite morphologic and certain biochemical similarities among such cell lines, the ability of small inocula to survive and grow in the hamster cheek pouch is a characteristic confined largely to cell lines derived from neoplastic tissue; conversely, the inability of such small inocula to survive and grow in the hamster cheek pouch is a characteristic of cell lines derived from non-neoplastic adult (but not necessarily embryonal) tissue. Additional differences in the behavior of cell lines derived from normal and neoplastic tissue have been reported previously (*16, 85*); unlike the frankly invasive, transplantable tumor produced in the hamster cheek pouch by cell lines of neoplastic origin, the nodules resulting from the implantation of 1.0×10^5 or more cells of non-neoplastic orgin are: 1) noninvasive, 2) regress rapidly, and 3) are not retransplantable. The characteristic histologic differences between such implants of cell lines of neoplastic and non-neoplastic origin also have been described elsewhere (*16, 85*). Certain of these differences in biological behavior also have been observed in conditioned rats even when implanted with large numbers of cells (*53, 57, 58*), and homotransplant studies in man suggest a similar differential transplantability of cell lines of normal and neoplastic origin (*53, 55–58*).

The experimental system described herein not only distinguishes between cell lines of neoplastic and non-neoplastic origin but, irrespective of the ultimate fate of the inoculum, also discriminates among cell lines within these broad categories through their differential implantability at differing inocula. As indicated in text-figure 1, all cell lines irrespective of origin may, on this basis, be arranged in a continuum of transplantability ranging from that observed with cells of adult normal or embryonal origin (*e.g.*, CCRF-202, -209, etc., text-fig. 1), which do not grow at 1.0×10^6 cells, and those of normal adult or embryonal origin (*e.g.*, L, CCRF-201, etc., text-fig. 1), which failed to grow at 1.0×10^5, to cells of neoplastic origin (*e.g.*, KB, HeLa, text-fig. 1), which grow at about 10 cells in the hamster cheek pouch. This continuum of response suggests (among other things, *vide infra*) the possibility that at inter-log doses the distinction between cells of normal and neoplastic origin may not be so abrupt, and that the apparent dichotomy in transplantability may indeed be a continuum around the critical dose of 1.0×10^4 cells. It is of further interest that a similar continuum of response is evident in cortisone-conditioned hamsters, and with cell lines derived from animal sources (text-fig. 1).

Since present knowledge of transplant systems indicates that the success or failure of a transplant is primarily a function of acquired immunity to graft antigens (*86–93*), the differential heterotransplantability of the mammalian cell lines considered herein may be attributable to differences in antigenic constitution. The distinction between normal and neoplastic cell lines in this system, or even between cell lines within each of these broad categories, may be due either to the presence (*94–100*) or absence (*101–107*) of qualitatively different antigens, or to a regular, quantitatively



HUMAN CELLS
NORMAL HAMSTERS

CELL LINE *	TISSUE of ORIGIN **	NO. CELLS IN INOCULUM 10^6	10^5	10^4	10^3	10^2	10
KB HELA	M,A M,A	●	●	●	●	●	●
J-III	M,A	●	●	●	●	●	○
CCRF-6 INTESTINE-407	M,A N,E	●	●	●	●	○	○
DETROIT-52	N,A	●	●	●	○	○	○
C, CCRF-204, 205,213, DETROIT-173 B	N, A	●	●	○	○	○	○
L, CCRF-201,206, 207, 211, 212	N,A	●	○	○	○	○	○
CCRF-202, 209,214	N,A	○	○	○	○	○	○

HUMAN CELLS
CORTISONE-CONDITIONED HAMSTERS

CELL LINE *	TISSUE of ORIGIN **	NO. CELLS IN INOCULUM 10^6	10^5	10^4	10^3	10^2	10
KB HELA	M,A M,A	●	●	●	●	●	●
J-III LLC-HE-I	M,A N,E	●	●	●	●	●	○
CCRF-106, INTESTINE-407	N,E	●	●	●	●	○	○
DETROIT-52	N,A	●	●	●	○	○	○
CCRF-200 LIVER-407	N,A N,E	●	●	○	○	○	○
CCRF-211, 212 MAF-83, CCRF-215	N,A N,E	●	○	○	○	○	○
CCRF-202, 209, 214	N,	○	○	○	○	○	○

ANIMAL CELLS
NORMAL HAMSTERS

CCRF-91	M,A	●	●	●	●	●	○
CCRF-101	M,A	●	●	●	●	○	○
CCRF-219	N,E	●	●	●	○	○	○
LLC-M-1	N,A	●	●	○	○	○	○
CCRF-216 CCRF-218	N,A N,E	●	○	○	○	○	○
BOVINE KIDNEY BOVINE SKIN+MUSCLE	N,E	○	○	○	○	○	○

ANIMAL CELLS
CORTISONE-CONDITIONED HAMSTERS

CCRF-91, 101, 180	M,A	●	●	●	●	●	○
CCRF-1534, Ca 755	M,A	●	●	●	●	○	○
CCRF-219	N,E	●	●	●	○	○	○
L-929 CCRF-208	N,A N,A	● ●	● ●	◉ ○	○ ○	○ ○	○ ○
CCRF-217 CCRF-218	N,A N,E	●	○	○	○	○	○
BOVINE KIDNEY BOVINE SKIN+MUSCLE	N,E	○	○	○	○	○	○

* Cf. Tables 1-4.
** M = Malignant; N = Non-Malignant; A = Adult; E = Embryonal.
● Growth.
○ No Growth.
◉ Grew at 1.0×10^4 cells in some experiments. Cf. Table 4.

TEXT-FIGURE 1.—Differential heterotransplantability of mammalian cells to the hamster cheek pouch.

differing distribution of the same antigen or complex of antigens (*85*, *108–110*). Such differences may be both qualitative and quantitative, neoplastic cells being characterized by the absence or modification of antigen present in normal cells (*111–114*). The continuum from "normal" to "neoplastic" among these differentially implantable cell lines suggests that the acquisition of neoplastic properties may be related to a progressive loss of antigen, which is reminiscent of the "*en bloc*" loss or modification (*111*, *115*, *116*) of genetic requirements, and the "antigenic simplification" (*91*, *111*) elucidated in homotransplant systems, as well as what has been described as the "stepwise" evolution of neoplasia (*117*), as opposed to the somatic mutation theory of oncogeny (*118*). The continuum of response at different dosages throughout the spectrum from normal to neoplastic cell lines further suggests the additional hypothesis that putative transitional stages between normal and neoplastic cells may be identifiable as different antigenic types through their differential heterotransplantability in the hamster, provided that such cell types can be fixed as reasonably stable populations in cell culture.

That a spectrum of autonomy indeed does exist *in vitro* among cell lines of neoplastic derivation is evidenced from the present experiments. That neoplastic cells *in vivo* manifest a similar variable autonomy requires no further clinical evidence than to call attention to the variation in the biological behavior of tumors (*119, 120*), and no further experimental evidence than the demonstrable distinction between the strain-specific and nonspecific neoplasms in mice (*111*) or the demonstration of an orderly progression from dependence to complete autonomy in certain experimental tumors (*121–128*). Differing degrees of autonomy among neoplastic cells also must be considered as an alternate explanation for the anomalous result obtained in the hamster cheek pouch with CCRF-100, -104, -105, -107, -108 (table 3) and Detroit-6 and -116P (table 6), rather than failure to isolate neoplastic cells *in vitro* because of the nature of the tumor specimen from which the cell lines were derived. However, isolation of apparently non-neoplastic cell lines from neoplastic tissue has been reported previously (*39, 129*) and, as pointed out by Berman *et al.* (*4*), unless it is assumed that all cells in a biopsy specimen of neoplastic tissue are indeed neoplastic, there is no way of knowing that the cells selected by culture *in vitro* are neoplastic, even if such cell lines were to be derived from single cells. In either case, it is evident that biological behavior in the hamster cheek pouch cannot always be predicted from the nature of the tissue from which a given cell line was derived.

With respect to the cell lines of normal origin used in this study, the classical view that autonomy is reflected in an escape from host-versus-graft responses implies that these cell lines, which possess a variable ability to implant and survive in the hamster cheek pouch at inocula in excess of 1.0×10^4 cells, possess variable degrees of autonomy. This variable heterotransplantability may not be merely an attribute of normal cells in culture, but may reflect a variation *in vivo* in antigenic structure or functional differentiation, permitting the existence of cell prototypes which range from those completely responsive to host-imposed restrictions to those which exhibit a degree of autonomy approaching that of the classical neoplastic cell. That variable autonomy may exist among normal cells *in vivo* is suggested by clinical-morphologic evidence of a continuum between reactive hyperplasia and neoplasia (*4, 119, 130*), by instances of inflammatory or reactive response in which unusual cells of abnormal morphology are present (*4, 131, 132*), and by those instances in which tumors presenting the clinical aspects of "cancer" are composed largely of what appear to be cells of normal morphology (*4, 119, 130*). Biological evidence of variability among normal cells is suggested by differences in ploidy in mammalian (*45, 133*) and plant tissue (*134, 136*), and by the varying degrees of immunological potency (*137, 138*) and competence (*138, 139–144*) in different stages of embryologic and postnatal development. That the biochemical factors responsible for this variable autonomy of normal cells may not necessarily be the same as those concerned with neoplasia is indicated in these experiments by the specific ability of cells of neoplastic origin to invade, grow progressively, and be serially transplantable in their heterologous hosts.

Whether normal cells exist *in vivo* with inherent variations in autonomy, or whether they exist at a given level of dependence but with a variable *propensity* for autonomous survival and are thereby selected by cultivation *in vitro*, does not affect the implication that all cell lines do not acquire neoplastic properties consequent to culture *in vitro*, as is evidenced by the present demonstration that cell lines of neoplastic and non-neoplastic origin can be distinguished from each other in an *in vivo* system. The spectrum of autonomy indicated herein, embracing cell lines of normal (non-neoplastic) as well as neoplastic origin, of course, implies that cells in culture certainly, and possibly their counterparts *in vivo*, may exist neither as "malignant" nor "normal" in the usual sense, but as either or both, "autonomy," "malignancy," and "growth potential" being variable properties of cell populations expressed relative to their environment. What these distinctions may ultimately mean in terms of autonomy or "malignancy" in man cannot be predicted, despite the reported suggestive evidence of a similar differential response with certain cell lines in humans (*53, 55–58*). Nevertheless, the biologic differences between serially propagated cell lines observed in these studies imply differences in the biochemical attributes of such cell lines, despite the still unanswered questions as to the nature of such biochemical differences.

SUMMARY

The heterotransplantability of 63 cell lines, established *in vitro* from human and animal normal and neoplastic sources, has been determined by implantation of quantitated inocula in the cheek pouches of Syrian hamsters (*Mesocricetus auratus*). With few exceptions, the hamster distinguished between cell lines of normal and neoplastic origin, despite the striking similarities between such cell lines *in vitro*. Cell lines of neoplastic origin survived and grew after implantation of 1.0×10^4 or fewer cells in the cheek pouches of unconditioned or cortisone-conditioned hamsters, whereas cell lines derived from adult non-neoplastic tissue, in general, did not survive and grow after implantation of similar inocula. The cell lines of embryonal origin included in these studies were variable; some failed to grow, while others were indistinguishable from cell lines of neoplastic origin in the hamster cheek pouch.

Despite the resistance elicited by implants of 1.0×10^4 cells of normal origin, and the failure of similar inocula of cells of neoplastic origin to elicit such resistance in the hamster, a continuum of response, with subtle gradations, between normal cell lines which failed to grow, even after implantation of 1.0×10^6 cells, to neoplastic cell lines which grew progressively from inocula of *circa* 10 cells, was evident among the cell lines

considered. The data suggest the possibility that at inter-log doses, the distinction between cell lines of normal and neoplastic origin may not be so abrupt, and that the dichotomy in "growth potential," or autonomy, might be converted to a continuum around the critical dose of 1.0×10^4 cells.

The most logical hypothesis to be adduced from the observed differential heterotransplantabilities of the cell lines considered is that cell lines differ from one another antigenically, or in degree of antigenicity; and that the hamster, by virtue of its unique response to tissue grafts, can distinguish among them.

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Plate 16

Figure 1.—Everted cheek pouch of Syrian hamster (*Mesocricetus auratus*).

Figure 2.—Cross section of cheek pouch. Hematoxylin and eosin. × 210

Figure 3.—Intradermal implantation of mammalian cell culture inocula into upper layer of epithelium of everted pouch.

Figure 4.—Intradermal bleb resulting from properly implanted inoculum.

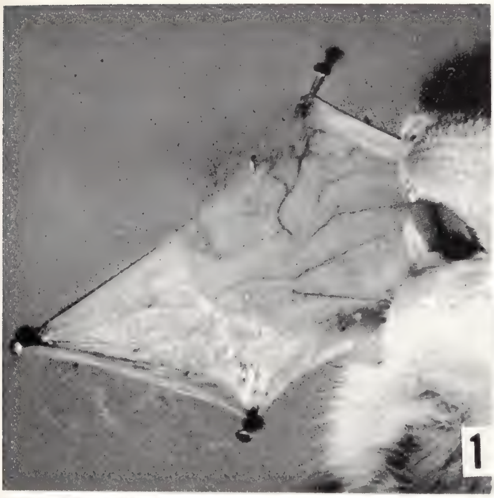

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PLATE 17

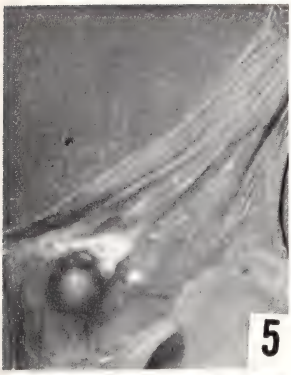
Characteristic heterograft response after implantation of serially propagated cell lines of neoplastic origin in the hamster cheek pouch.

FIGURES 5 AND 6.—J-111 (*cf.* table 2), 1.0 × 10^5 cells in normal hamsters, 21 and 28 days (same pouch) post implantation.

FIGURES 7 AND 8.—J-111, 1.0 × 10^4 cells in cortisone-conditioned hamsters, 14 and 21 days (same pouch) post implantation.

FIGURES 9 AND 10.—J-111, 1.0 × 10^3 cells in cortisone-conditioned hamsters, 14 and 21 days (same pouch) post implantation.

FIGURES 11 AND 12.—HeLa (*cf.* table 2), 1.86 × 10^4 cells in cortisone-conditioned hamsters, 14 and 34 days (same pouch) post implantation.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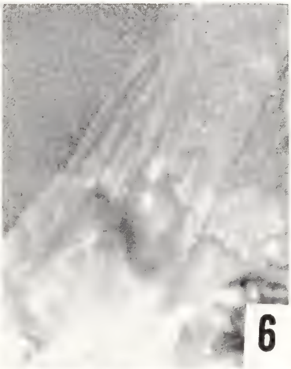
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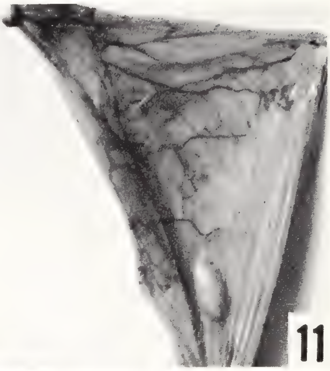
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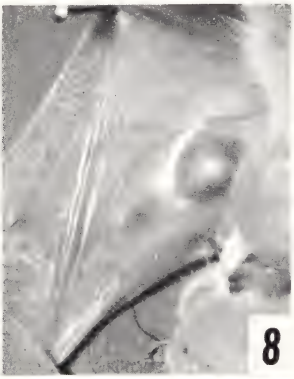
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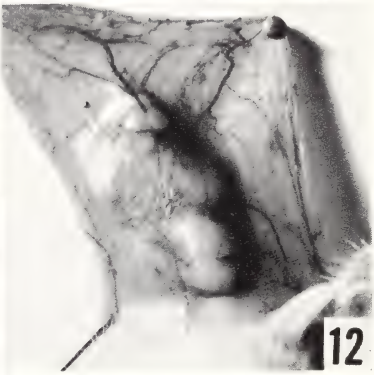
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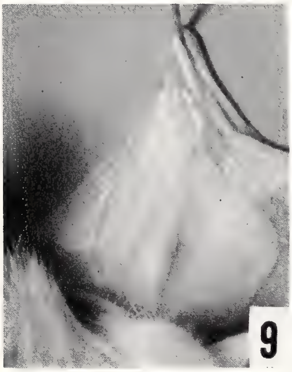
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PLATE 18

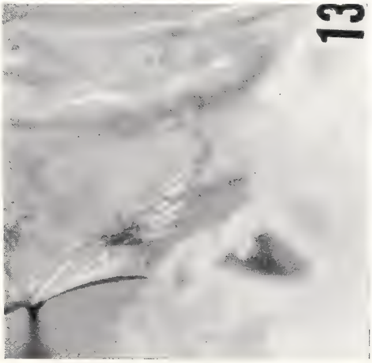
Characteristic heterograft response after implantation of serially propagated cell lines of normal origin in the hamster cheek pouch.

FIGURE 13.—Detroit 173B (*cf.* table 2), 1.0 × 10^5 cells in cortisone-conditioned hamsters, 7 days post implantation.

FIGURES 14, 15, AND 16.—Same cheek pouch, 14, 21, and 28 days post implantation.

FIGURE 17.—Detroit 173B, 1.0 × 10^5 cells in normal hamsters, 7 days post implantation.

FIGURES 18, 19, AND 20.—Same cheek pouch, 14, 21, and 28 days post implantation.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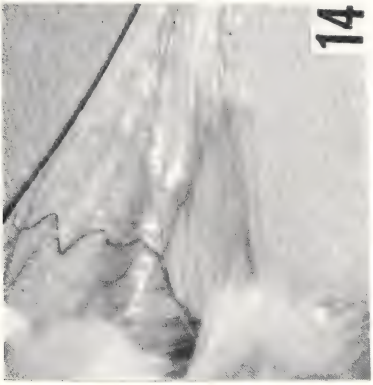
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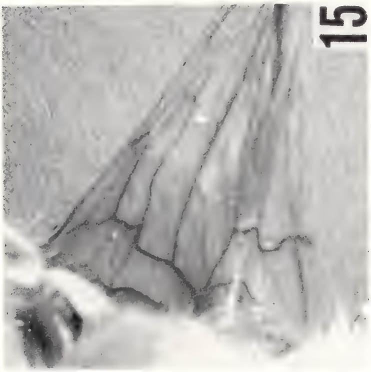
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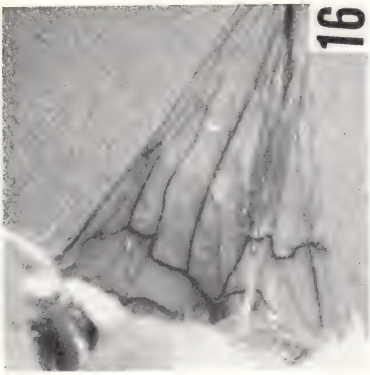
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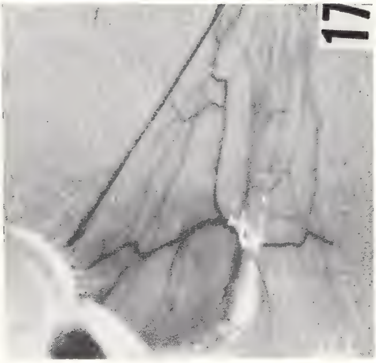
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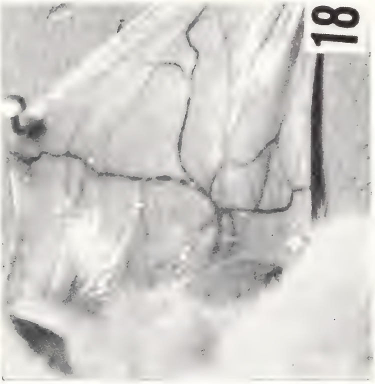
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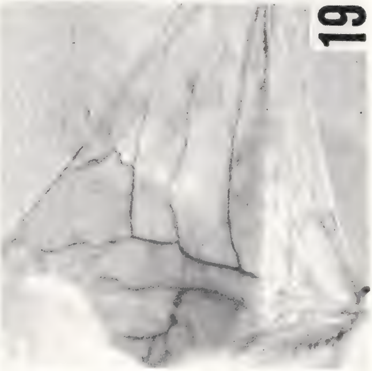
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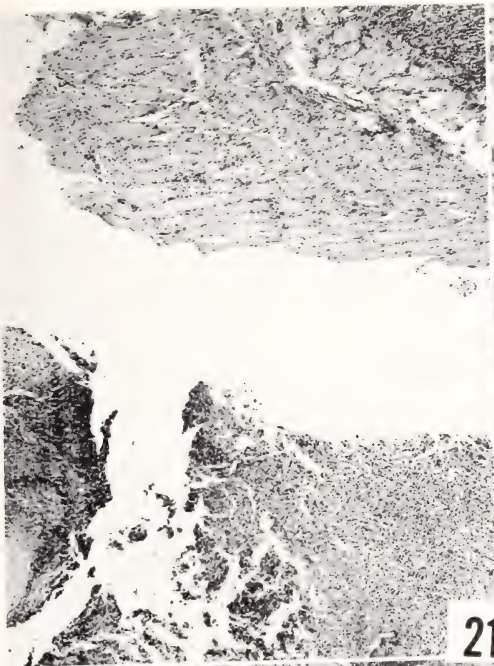
PLATE 19

FIGURE 21.—Section of biopsy from which CCRF-107 was isolated *in vitro.* Hematoxylin and eosin. × 90

FIGURE 22.—Section of biopsy from which CCRF-108 was isolated *in vitro.* Hematoxylin and eosin. × 30

FIGURE 23.—Section of biopsy from which CCRF-100 was isolated *in vitro.* Hematoxylin and eosin. × 575

FIGURE 24.—Section of nodule in cheek pouch of normal hamsters 6 days after implantation of 1.0×10^6 cells of CCRF-104. Hematoxylin and eosin. × 160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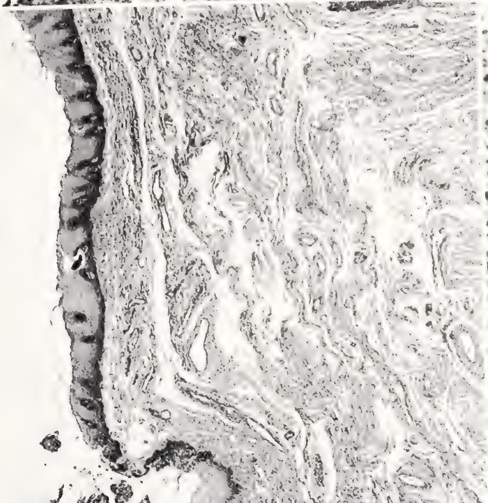
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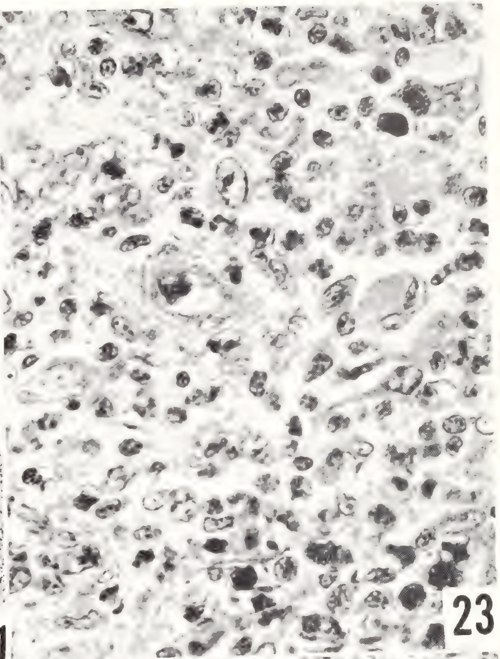
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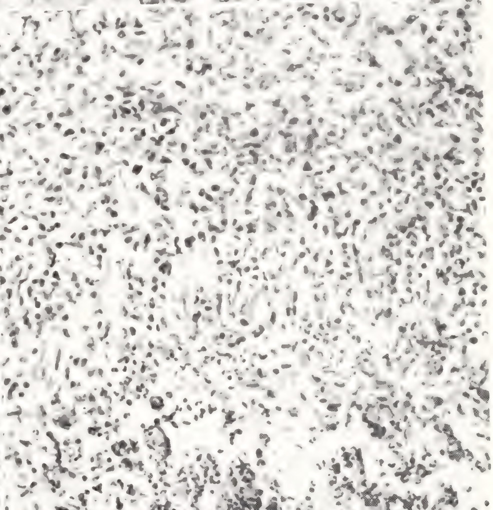
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DISCUSSION

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No manuscript was submitted.

Deoxyribonucleic Acid, Virus, and Protein Synthesis in Animal Cell Cultures [1, 2]

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5-FLUORODEOXYURIDINE (FUDR) is a noncompetitive inhibitor which blocks the formation of thymidylic acid (*1*). As a consequence of this action, it is an effective inhibitor of the synthesis of deoxyribonucleic acid (DNA). It has proved a valuable tool in evaluating the role of DNA metabolism in poliovirus and vaccinia virus replication. Further, it has yielded data that suggests a mechanism for the control of the levels of certain enzymes in the cell.

THE ROLE OF DNA SYNTHESIS IN POLIOVIRUS REPLICATION

In previous studies with poliovirus-infected HeLa cells, we observed that, as a consequence of infection, there was an almost complete and immediate inhibition of ribonucleic acid (RNA) and DNA synthesis even though the infected cells were contained in a medium which permits optimal rates of RNA and DNA synthesis in uninfected cultures (*2*). These observations were based on colorimetric procedures which are not sufficiently sensitive to permit a decision as to whether the inhibition of DNA synthesis was partial or complete. The uptake of cytidine-2-C^{14} into the nucleic acids was studied as a more sensitive indicator of low levels of nucleic acid synthesis. Uptake of the radioactive precursor into DNA was observed during the first 6 hours of the infective cycle (table 1). The extent of DNA synthesis was calculated to be a 5 percent increment, an amount too small to have been detected as a net change by colorimeter methods. These data suggested that low levels of DNA synthesis might be necessary for poliovirus replication even though this virus does not contain DNA. To investigate the possible role of DNA in poliovirus replication, we used the metabolic inhibitor 5-fluorodeoxyuridine (FUDR). Its effects on uninfected HeLa S3 cultures are seen in table 2. Inhibitor concentrations of 5×10^{-7} M or higher prevent any detectable net synthesis

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[1] Presented at the Syverton Memorial Symposium on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

[2] These studies were made possible through the kindness of Dr. Robert Duschinsky of Hoffman-La Roche, Inc., Nutley, N.J., who generously provided us with fluorouracil-2-C^{14} and 5-fluorodeoxyuridine-2-C^{14}.

[3] Laboratory of the Biology of Viruses, National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Table 1.—Uptake of cytidine-2-C^{14} into the DNA pyrimidine bases of polio-infected and uninfected growing cell cultures*

Time after infection (hours)	Specific activity of the DNA pyrimidine bases (cpm/μmole)			
	Growing uninfected culture		Polio-infected culture	
	Cytosine	Thymine	Cytosine	Thymine
3	3, 420	3, 500	2, 500	2, 500
6	10, 100	10, 600	4, 410	4, 610
9	18, 900	14, 200	4, 430	4, 800
12	26, 400	27, 900	5, 710	5, 820

*For experimental details *see* (*2*).

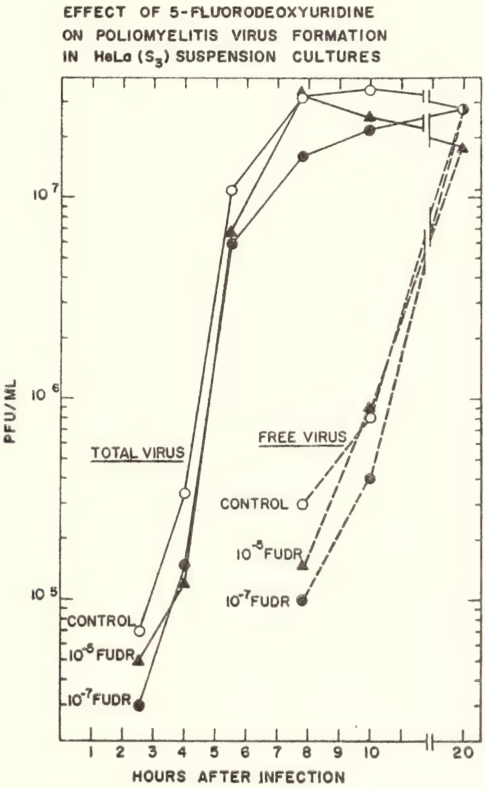
in DNA as measured by the diphenylamine reaction. Further, when concentrations of FUDR of 10^{-6} M and uridine-2-C^{14} at a specific activity of 536,000 cpm per μmole and a concentration of 2×10^{-5} M were simultaneously added to HeLa suspension cultures, no counts were detected in DNA thymine. In a control culture in this same 2-hour period, the specific activity of DNA thymine was 6550 cpm per μmole. From this it can be concluded that the inhibition of thymidylic acid synthesis by FUDR is both complete and immediate. Further, the quantities of thymine derivatives in the acid-soluble fraction of the cell are not sufficient to permit DNA synthesis to occur.

When the effects of FUDR on poliovirus replication were measured, it was clearly demonstrated that the rate of virus replication was in no way affected, even though DNA synthesis was completely inhibited (text-fig. 1). In this experiment, FUDR was added to cells 3 hours prior to infection and was present during adsorption of virus to cells and throughout the period of virus replication. These experiments demonstrate that a ribonucleoprotein can replicate, presumably with no information for this synthesis contained in the DNA. A similar demonstration of the noninvolvement of DNA in poliovirus replication has been obtained by the use of aminopterin (*3*).

Table 2.—Effect of FUDR on HeLa cell metabolism

Molar concentration of FUDR	Fold increase in 24 hours*		
	DNA	RNA	Protein
10^{-5}	0. 98	1. 30	1. 53
10^{-6}	1. 01	1. 46	1. 46
5×10^{-7}	1. 08	1. 51	1. 59
10^{-7}	1. 29	1. 60	1. 67
5×10^{-8}	1. 39	1. 69	1. 79
10^{-8}	1. 51	1. 73	1. 79
5×10^{-9}	1. 68	1. 92	1. 90
10^{-9}	1. 84	1. 99	2. 00
Control	1. 90	1. 91	1. 97

*Samples were analyzed at 0 time and then again at 24 hours. Values for RNA, DNA, and protein at 0 time are set equal to 1, and the results obtained at 24 hours are expressed as the fold increase relative to the 0 time values.



TEXT-FIGURE 1.—FUDR at the molar concentrations indicated was added 3 hours prior to infection and was present throughout the infective cycle.

EFFECTS OF FUDR ON VACCINIA VIRUS REPLICATION

When a concentration of FUDR which completely inhibits DNA synthesis in uninfected cells is added to HeLa cells infected with vaccinia virus, there is a complete inhibition of virus synthesis (table 3). FUDR inhibits only the synthesis of thymidylic acid, but does not effect its in-

Table 3.—Inhibition of vaccinia virus production by FUDR and its reversal by thymidine*

Molar concentration of FUDR	Molar concentration of thymidine	Virus titer (pfu/ml)†	
		3.7 hours	24 hours
—	—	2.4×10^5	2.6×10^7
10^{-6}	—	2.4×10^5	1.5×10^5
10^{-6}	10^{-5}	4.0×10^5	2.0×10^7
10^{-6}	10^{-6}	—	2.0×10^7

*Cells were infected, washed free of unadsorbed virus, and resuspended at a final cell population of 2×10^5 cells per ml. Additions as indicated above were then made. Titrations were done on chick-embryo monolayers with the use of the plaque assay.

†Plaque-forming units.

corporation into DNA (*1, 4*); thus the inhibition of vaccinia virus formation is reversed by the addition of thymidine (table 3).

The ability of an RNA virus to replicate in the presence of FUDR and the inhibitory effect of FUDR on the DNA-containing vaccinia virus suggested its usefulness as a method for determining whether a virus contains RNA or DNA (*5*). Other viruses that have been examined by this procedure tend to confirm this suggestion (*6, 7*).

EFFECT OF FUDR ON UNINFECTED CELL CULTURES

In addition to its primary effect on thymidylic acid synthesis, FUDR is converted to the free base, 5-fluorouracil (FU), and this compound in turn is converted to the 5′-ribonucleotide, 5-fluorouridylic acid (FUMP) (*4*). FUMP in some systems can partially inhibit the formation of uridylic acid (UMP), an RNA precursor, or it may compete with UMP, and itself become incorporated into RNA (*4*)—this incorporation of the analogue resulting in the formation of fraudulent and nonfunctioning species of RNA.

The effect of various concentrations of FUDR on uninfected HeLa S3 suspension cultures is seen in table 2. Those concentrations of inhibitor greater than 5×10^{-7} M which produce a complete inhibition of DNA synthesis still permit continued RNA and protein synthesis, though at suboptimal rates producing unbalanced growth, and finally loss of cell viability results similar to those previously reported (*8–10*).

At low concentrations of FUDR, in the range 10^{-7} to 10^{-8} M, not only is there a partial inhibition of DNA, but also of RNA and protein synthesis. The irreversible nature of the inhibition of thymidylic acid formation by FUDR explains its effectiveness in blocking DNA synthesis at these low concentrations. However, none of the mechanisms by which FU effects RNA seemed likely to function at these concentrations. What seemed the most likely explanation of this inhibition in the synthesis of RNA was that RNA synthesis was dependent on the synthesis of DNA.

The mechanisms by which a fluorouracil derivative could directly effect RNA have been examined. One possibility is that fraudulent species of RNA are formed as a result of the incorporation of FUMP into RNA, and that such species are nonfunctioning and interfere with further RNA synthesis. By direct examination of the degree of incorporation of FU-2-C^{14} into RNA it was observed that at 10^{-8} M, a concentration where a 15 to 25 percent inhibition of RNA was observed, there was an insignificant incorporation of the analogue; 0. 001 percent of the uracil being replaced by fluorouracil. Further, concentrations of uridine 50- to 5,000-fold in excess of the quantity of FUDR do not reverse the inhibition in the synthesis of RNA. However, exogenous thymidine which is incorporated only into DNA thymine completely reverses the inhibition in the synthesis of both RNA and DNA, a possible indication of their coupled synthesis, and confirmation that the inhibition of RNA synthesis was not a direct effect, but was caused solely by the inhibition in the synthesis of DNA.

An examination of subcellular fractions in inhibited cultures reveals that when DNA synthesis is inhibited, the synthesis of that RNA and protein which occurs in the nucleus is also inhibited. However, there is no effect on the rate of formation of cytoplasmic RNA and protein (table 4). These data suggest that the formation of nuclear RNA and protein is dependent on the synthesis of DNA. No such dependence is displayed by cytoplasmic RNA and protein.

Table 4.—Effect of FUDR on the synthesis of RNA, DNA, and protein*

Molar concentration of FUDR	Fold increase in 20 hours†				
	Nucleus			Cytoplasm	
	DNA	RNA	Protein	RNA	Protein
10^{-6}	1.02	.964	1.28	1.81	1.68
10^{-7}	1.09	.994	1.30	1.75	1.72
10^{-8}	1.61	1.43	1.72	1.71	1.73
—	1.88	1.73	2.23	1.72	1.72

*Cells were fractionated with citric acid according to Dounce's procedure as described by Newton and Stoker (*11*).

†*See* footnote table 2.

That the synthesis of certain species of protein (and presumably certain enzymes) is dependent on the synthesis of DNA suggests a mechanism for the regulation of enzyme levels in growing and resting cultures. Nuclear and cytoplasmic enzymes are degraded under growing and nongrowing conditions as a result of protein turnover (*12*). Under growing conditions, nuclear, as well as cytoplasmic enzymes, which are degraded by turnover, can also be resynthesized, for during growth there is synthesis of DNA. Under nongrowing conditions, however, nuclear enzymes that are degraded as a result of turnover cannot be reformed in the absence of DNA synthesis and will be lost from the cell, while cytoplasmic enzymes can be resynthesized and will thus maintain their level in the cell. Thus, with the discontinuance of DNA synthesis, there will be a decrease in or disappearance from the cell of a group of enzymes.

SUMMARY

Data relating to the role of DNA metabolism in poliovirus and vaccinia virus-infected cells have been presented. Further, evidence suggesting that the levels of nuclear enzymes may be dependent on the rate of DNA synthesis has also been cited.

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DISCUSSION

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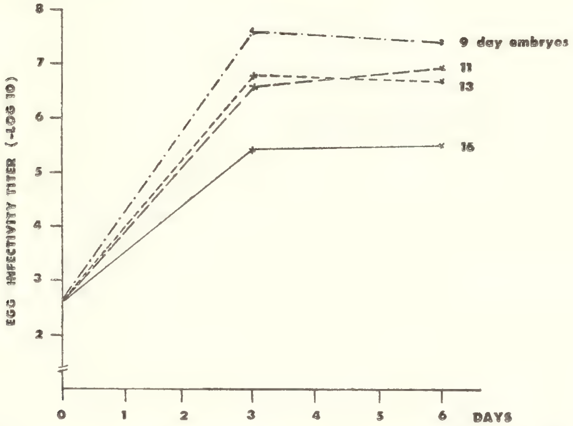
Dr. Salzman's excellent discussion of the effect of inhibitors on virus replication and on cell metabolism reveals how much our understanding of the interrelationship between the synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein has advanced in the last few years. From such studies and those of Maassab and Ackermann (*1*), Ginsberg and Dixon (*2*), and other workers in this field (*3*), the picture of what happens to a cell, in response to external factors, is becoming well documented. However, as Ackermann (*4*) has pointed out, the question of why it happens or, more important, why it happens in some cells and not in others, or at some times and not at others, is still far from clear.

Many factors must be involved and, in commenting on Dr. Salzman's paper, I would like to mention just one, that of cell age. Earlier in this symposium, several speakers have discussed the effects of strain age in months or years. It should not be forgotten that the age of the cells in hours or days can also be a critical factor, even if less dramatic than the long-term effects. This cell age can be regarded as the "developmental" age of the cell.

Since tissue cultures are prepared from the full spectrum of ontogenesis, the physiological age of the tissue at the time of explantation must be considered. However, the alterations in internal milieu, which occur as tissue ages, are under intensive investigation by embryologists and physiologists, and I would only like to illustrate with one example the effect which this aging has on experiments *in vitro*. Text-figure 1 (*5*) shows the result of inoculating PR8 influenza virus into chorio-allantoic membrane fragments suspended in synthetic medium M 150. It can be seen that the

9-day-old tissue permitted the development of a higher viral titer than did older tissues. This type of relationship between tissue age and its ability to support virus growth has been observed by most virologists, and in most cases "the younger the better" seems to be the rule.



TEXT-FIGURE 1.—Viral titer after inoculation of PR8 influenza virus into chorio-allantoic membrane fragments.

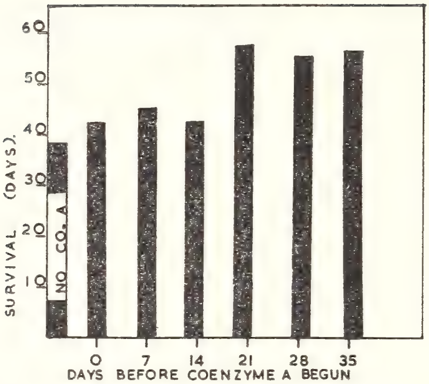
The second age factor, that of age *in vitro,* is much less predictable. Everyone selects cultures at a particular stage in development for a particular purpose, and Dr. Salzman emphasizes that he uses cells in logarithmic phase. Selection is necessary, but it does not reflect the whole picture. For example, if enzyme induction studies are carried out in a chemostat, a steady state of enzyme production can be achieved, whereas, if the cells are allowed to grow through lag, logarithmic, and finally to stationary phase, it can be shown that there is a particular point in the developmental cycle which is optimum for the production of a given enzyme.

It is difficult to show this type of behavior in cell strains because, so often, when the population is maintained high enough to give readily measurable changes, the rate of growth is too rapid to show all the phases.

On the other hand, when fresh tissue explants are maintained *in vitro* under conditions that do not force rapid cell division, these age effects can be seen. Text-figure 2 illustrates this type of effect. The tissue was fresh chick-embryo-heart fragments surviving in synthetic medium M 150. These fragments live about 38 days and follow a metabolic pattern of a slow lag phase, then a very active phase (corresponding to logarithmic phase in growing cells), and finally a declining phase. These patterns have been shown in our laboratory by chromatographic analysis of the fluids removed at different intervals (*6*).

Text-figure 2 shows the effect of the addition of coenzyme A (0.25 mg/L) to the medium at different intervals after explantation. In young cultures, up to 2 weeks old, the response to this level of coenzyme A is negligible. However, if the cultures are allowed to age in M 150 for 21 days or more before the coenzyme is added, they show a definite response by an increased survival of 17 to 21 days. This is not simply added coenzyme counteracting a progressive depletion of the original tissue reserves. If it were, all tissues would live the same length of time. In fact, in the case of cultures that received coenzyme at 35 days, it would appear to be a virtual "rejuvenation" because without coenzyme A they would have died at an average of 38 days, as did the controls. Therefore, by 35 days, when they first received coenzyme A, much of the tissue must have been moribund yet they revived and lived a further 18 days.

The difference in response of the cultures in text-figure 2 would seem, therefore, to reflect the difference in age or developmental stage of the tissue at the time that it



Text-figure 2.—Survival of fresh tissue explants *in vitro* after addition of coenzyme A to medium.

received coenzyme A. Such illustrations of the effect of one nutritional factor suggest that if many others are studied in relation to cell age, if we can analyze the developmental stage of a cell by means such as Dr. Westfall indicated, we may then, by knowing the internal state of the cell at that particular stage, be able to show *why* it can react to virus or other external factors, with the same precision with which Dr. Salzman has shown *what* reactions occur. To do this we must look not only at the long-term alterations that occur in cell strains, but also at the changes which occur in each phase of growth.

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Some Morphologic Variations of Certain Cells Under Controlled Experimental Conditions[1]

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OF RECENT years, the rapid advances in the study of cultures of animal tissue cells have resulted in many workers considering the study of cell morphology about as out of date as the age of dinosaurs. In spite of that, however, I am going to describe certain changes in cell morphology as we have observed them in our laboratory.

In our early work, an attempt was made to keep records of cell morphologic changes and general culture architecture over an extended period by written cell descriptions. This method proved to be entirely impractical for the achievement of any reasonable accuracy, therefore a system and equipment were developed for photographing a significant and representative area, 1.00 × 1.25 mm, of each culture at a magnification of 200, on an 8 × 10 inch negative. The objective used was a 16 mm e.f. Zeiss apochromat, N.A. 0.30 in conjunction with a #II Zeiss Homal ocular, and with a projection distance of approximately 125 cm.

In this recording, a deliberate attempt was made to use a fine grain, high-contrast film,[3] to cover a statistically significant and representative area of the culture, and to obtain as good resolution as practical for living cells growing in a good optical quality blown Pyrex #7740 glass culture flask, such as the Carrel D-3.5 flask. An effort was made to have reliable equipment, which would keep the culture at 37.5° C and at the same time be able to take records of at least 100 to 140 cultures per day under uniformly controlled conditions. No prints were made except when necessary for printed reproduction; instead an illuminated viewing unit, able to handle 84 negatives simultaneously, was used for examination and comparison of the negatives. This was necessary to study the full detail of the negative, some of which is always lost in the print.

Cultures were photographed usually at a relatively constant age and at least 8 hours after cells had been subjected to the shock of fluid change. Before recording, cultures were subjected to as little chilling or shaking as possible. The light used was kept minimal and was passed through a heat filter and a filter to eliminate ultraviolet and far visual wavelengths. Light intensity was controlled by a variable transformer in the light circuit so as not to change resolution of the optical system by changing the active numerical aperture of the objective.

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[1] Presented at the Syverton Memorial Symposium on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

[3] Kodak contrast process panchromatic film, developed 8 to 10 minutes at 72° F in modified Eastman DK76 containing 30 g Kodalk per liter.

After more than 18 years of use, this camera (*1*), with only certain facilitating modifications, and the general methods outlined, still constitute the central cell morphology recording unit of our laboratory. While cell culture types have changed with time, the general concepts and methods of recording general cell and culture morphology have continued essentially unchanged. In particular, we still attempt to obtain comparable photographic records with as little agitation or chilling of the cells as possible, and in the original culture flasks in which they were grown.

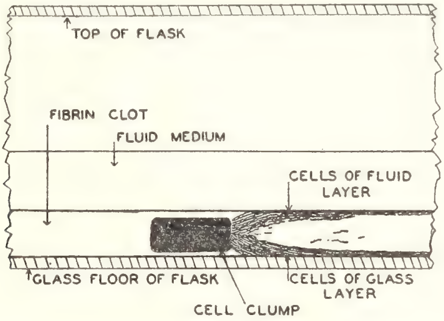
To date more than 17,000 negatives have been made. The system allows us to compare and verify cell changes over an extended period of years and, in instances, to recognize and validate changes in morphology which would never have been recognized from any practical written description.

More recently, data obtained from these still photographs are being supplemented by information from comparative cinematographic studies (*2*). With these, also, an attempt is made to carefully control experimental conditions.

During the course of an extended study of the action of an extremely low concentration of 3-methylcholanthrene on cells of a strain of normal mouse subcutaneous fibroblasts, the cells were grown in Carrel flasks in a dilute plasma matrix with a supernatant fluid medium overlay (*3–5*). The fibroblasts at different stages of treatment, and thereafter, showed the most complex group of morphologic changes we have ever attempted to follow in culture. This study could never have been made except by photographic records. Some of the changes observed are typical of normal fibroblast cultures; some arose from action of carcinogen on the cells. A number of illustrations showing changes seen in the fibroblasts will be presented from this series of studies, with illustrations of certain types of cell changes seen in later studies.

In such plasma matrix cultures as those used in the early carcinogenesis studies, fibroblasts tend to migrate out from the explant surface and to collect at the glass/plasma interface of the culture and in the plasma at the fluid interface (text-fig. 1). As the culture gets larger the morphology of cells at different distances from the explant may be radically different. With too small a culture, the more peripheral cells may die. Cells may develop tremendous fat globules with some media and more normal size explants. With normal size explants, cells closer in to the explant may continue to show a general spindle shape and may develop large numbers of neutral red-staining granules, while the most peripheral cells may become relatively tremendous in size, lose orientation, and develop a variety of bizarre forms (fig. 1). These variations in size and form of the peripherally situated cells seem clearly associated with the density of the cell population and the intimacy of cell contact with large volumes of medium to which the cells are incompletely adapted.

Morphology of cells at the glass and fluid interfaces is often radically different. In extreme instances, the difference is so great that the morphologic picture is hopelessly confusing unless the cells at both interfaces are studied separately. An instance of this in figures 2 and 3 shows typical morphology at the glass and fluid interfaces, respectively, of a carcinogen-treated culture. In figure 2 the carcinogen has caused cell cohesion so great that migration of the cells is tremendously reduced and the cell sheet appears typical of a massive epithelial sheet, or even a syncytium. Detailed study of such masses shows that the cells are not syncytial however, since on careful fixation the cells separate cleanly from each other. Apparently, due to this extremely strong cellular cohesion, migration of cells of such massive sheets is greatly retarded, though many mitoses are seen and the cinematograph shows a great deal of intracellular agitation. In figure 3, the elongated spindle-shaped cells in the plasma at the fluid interface, only a few tenths of a millimeter away, are entirely discrete, and, laterally, cleanly separated from each other. Cells of this fluid interface migrate much more rapidly. Each of these two interface cell forms is rapidly interchangeable if transferred to the other interface.



TEXT-FIGURE 1.—Diagram showing cells migrating out from an explant in plasma clot tend to collect at glass and fluid interfaces. This tendency is characteristic of plasma matrix cultures even when the plasma is only 0.2 or 0.3 mm thick.

Two other morphologic transformations observable most clearly at the glass interface of cells treated with a lighter dose of carcinogen are shown in figures 4 and 5. In the first of these the main axis of migration of the spindle-shaped fibroblast is much less clearly defined. At this stage the cells send out numerous lateral processes. Instead of traveling more or less in a straight line, the cells tend to shift their direction into one of these lateral processes, so that the long strands of cells arch over and join other strands, and so enclose within their loops small or large areas of plasma matrix. At a later stage, shown in figure 5, the cells become even more laterally coherent, more sheetlike, and appear somewhat similar to the thin sheets of cells so typical of certain epithelial cells in culture. This stage of the cell transformation at the fluid interface also shows the cells less spindle-shaped, more flattened, and sheetlike, but the change in cell morphology is less strikingly demonstrated than at the glass interface.

One remarkable morphologic cell transformation for which no explanation was ever found is shown in figure 6. Fibroblasts of the stage shown in figure 4 were injected into a mouse to form a tumor. Tissue from this typical sarcoma was retransferred to culture. Within a very short time practically all cells in the culture series had gone over into the tremendous epithelial-like cells shown. Only one of these cells was ever observed in mitosis and its nucleus contained so many chromosomes that their number could not be counted. Within a few transfer generations all of these tremendous cells had disappeared, apparently overgrown by the few remaining normal-size cells in the cultures.

In surface substrate cultures, such as a monolayer of cells, *e.g.*, NCTC clone 929 fibroblasts growing on glass, under a layer of fluid nutrient, such as a dilute serum-embryo extract-balanced saline mixture, the cell population density is often a major factor in influencing cell morphology. A scant population density with a relatively tremendous volume of fluid nutrient may result in cell death; a somewhat greater cell population density allowing the cells to survive demonstrates a typical fibroblast-like, fusiform, oval, or rounded shape adherent to the glass, as in figure 7. As cell population on the substrate is increased, lateral pressure of crowding by other cells often forces the cells into more or less polygonal or spherical shapes as shown in figure 8. Cinematographic studies show that such crowding also greatly reduces cell migration. Cells of clone 929 may heap up and become an increasingly thick layer until necrosis starts in such multicellular-thick areas.

Figure 2 shows extreme cell cohesion so extensive as to interfere greatly with cell migration. Cinematographic analysis of cultures of NCTC clone 929 cells shows sufficient cell cohesion to result in cell clumping and cohesion of loose cells to form clumps (fig. 9). Even in thinly populated cultures, adhesion of the cells to the glass substrate is limited, since many of the cells can be detached by shaking the culture by hand (*2*, *6*). With these cells their limited cohesion and even more limited adhesion to the glass substrate allows them to migrate rapidly. With strain NCTC 2071, derived from clone 929, and grown for more than 6 years in chemically defined medium to which no protein is added, adhesion to the glass substrate is so tight that the cells cannot be shaken loose (fig. 10). As the population density of the culture increases there is little evidence of cell cohesion; instead the cells become polygonal, flattened, and sheet-like (fig. 11). When crowding of the culture is extreme, so that there is a continuous monolayer, instead of forming cultures more than 1 cell layer in thickness, the cells have a great tendency to float loose into the supernatant fluid.

Even with thinly populated cultures of this strain on a glass substrate under chemically defined nutrient fluid medium NCTC 109, cinematographic analysis of cell behavior shows the cells so tightly adherent to the glass that cell migration is tremendously retarded. Instances are seen in which the terminal processes of an elongated fusiform cell are so tightly adherent to the glass that when it attempts to migrate it is unable to detach its posterior process from the glass. Consequently a wave of motion passes from the posterior to the anterior end of the cell, and there reverses itself and again passes toward the posterior end of the cell where the same process may be repeated over and over, with the cell in some instances never migrating so far as its own length in either direction. This occurs even in relatively thinly populated cultures in which the cell has an entirely adequate clear area around it for migration. However, while this adhesion to the glass does tremendously reduce migration, it does not interfere with cell proliferation by mitosis. Cell proliferation and population increase in these cultures are rapid.

These considerations have dealt with certain transformations in morphology of fibroblast-like mesenchymal cells. When we tried to grow human skin epithelium, repeated attempts gave proliferation among cells of a thin coherent sheet which migrated out from the explant as in figure 12. Repeatedly these cells were later overgrown by loose, peripherally situated fibroblast-like cells. Finally, by continuous study and, in 1956, by establishing clones of cells of the thin epithelial sheet, we clearly demonstrated that this epithelial sheet tended to break up peripherally into individual loose cells which on casual morphologic examination could be mistaken for fibroblasts (*7*).

This clone of human skin epithelium, cloned in 1956, and designated as NCTC clone 2544 is still growing rapidly. For the last 2 years these cells have been designated NCTC 3075 and have been maintained in chemically defined medium without any added proteinaceous medium or undefined hydrolysates. At the present time, the strain continues to show a definite tendency to lateral cohesion and sheetlike forms on a glass substrate at certain stages of the culture growth cycle, and continues its rapid proliferation. It grows luxuriantly in rapidly agitated fluid-suspension culture. Like the cells of strains 929 and 2071 its cells tend to be spherical in agitated fluid suspensions. However, in agitated fluid-suspension cultures, the cells have a greater tendency to cohere into clumps than do cells of clone 929 for instance. A similar tendency to clump is also seen with the HeLa cell, also of epithelial origin, in fluid-suspension culture.

Another epithelial cell strain that we have studied extensively is a strain of liver epithelium NCTC 721 from a 2-day-old mouse. This strain has now been maintained in culture since 1948 and was established as cloned strain NCTC 1469 in 1952.

The early morphology of the cells of strain 721 showed irregular epithelial sheets (fig. 13), laterally coherent with other comparable cells on all sides. Later the epithelial sheets broke up (fig. 14), and the nature of these cells became less typical of liver epithelium. In fluid-suspension culture, with the *addition of serum*, the growth in NCTC 109 was luxuriant, but for continued rapid growth the serum could not be omitted from either static substrate cultures or rapidly agitated fluid-suspension cultures (*8*). In the latter the cells are spherical and do not tend to cohere; they form an excellent suspension of individually isolated cells. Even yet, after these years in culture on stationary glass substrates in fairly young cultures, the cells have a tendency to assume sheetlike forms on the glass due to lateral cell cohesion, though in older cultures they often separate and become more spherical, as in figure 15.

Certainly not all cell strains or types can be assuredly recognized by morphologic studies alone. This cloned strain of liver epithelium is an excellent example of how recognition of a cell strain may, at the present state of our knowledge, depend on study of many parameters. Its cells still continue to form epithelial-like sheets at early stages of its culture growth though in later stages they tend to round up. They lay down glycogen (*9*); their nutritional requirements are different from those of the fibroblast-like cells studied; they support excellent growth of mouse hepatitis virus (*10*); on intramuscular injection into mice they have given rise to carcinoma (*11*), the cells of which suggest anaplastic liver epithelium; the cells show high arginase and rhodanese activity (*12*) and 4 detectable steroid reductases (*13*).

I would certainly not advocate morphologic studies alone for cell classification and characterization, but I do unequivocally urge morphologic studies under carefully controlled and defined conditions as an essential basic parameter in cell characterization.

SUMMARY

Radical morphologic changes in cells must necessarily reflect physiologic changes of greater or less magnitude. Most efficient use of morphology as one basic index in study of cell change or in cell-type identification often requires extremely accurate control of experimental conditions. Wide variations in morphology observed as a result of change in experimental conditions were discussed for a number of established cell strains. An attempt was made to explain some of the mechanisms involved in the changes.

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PLATE 20

FIGURE 1.—Fibroblasts toward center of culture (*bottom*) are more or less uniform in size, spindle-shaped, and radially oriented; peripheral cells are often much larger in size, more random in shape and orientation. × 200

FIGURE 2.—Fibroblasts of same original strain shown in figure 1, growing as massive lobulated cell sheet, at glass interface. × 200

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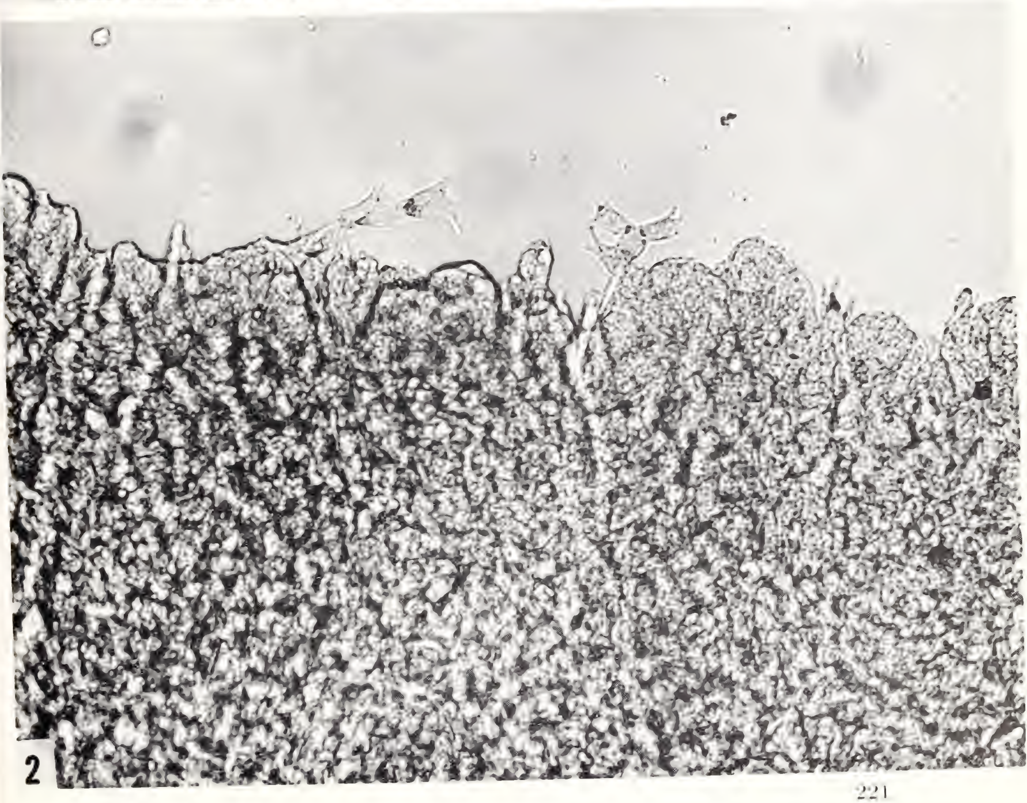

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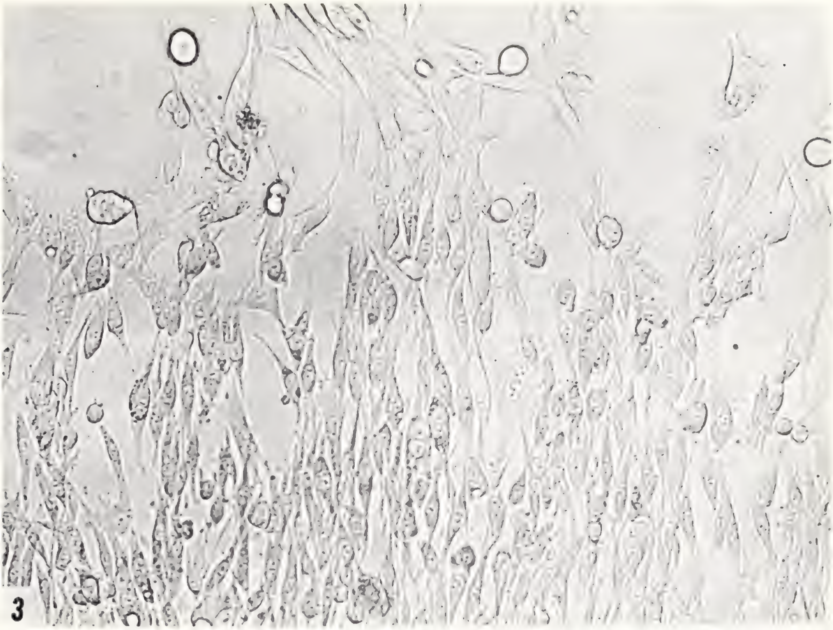
Plate 21

Figure 3.—Same type cells as figure 2, showing loose, laterally noncoherent cell growth typical of fluid interface for this cell strain. *Bottom* of picture is toward *center* of explant.

Figure 4.—Another type of fibroblast morphologic transformation. *Note* characteristic formation of irregular lateral processes of the cells and tendency of cells to lose radially oriented axis and divert migration into lateral processes, so enclosing islands of plasma and producing a "looping" type of culture architecture. *Bottom* is toward *center* of explant. × 200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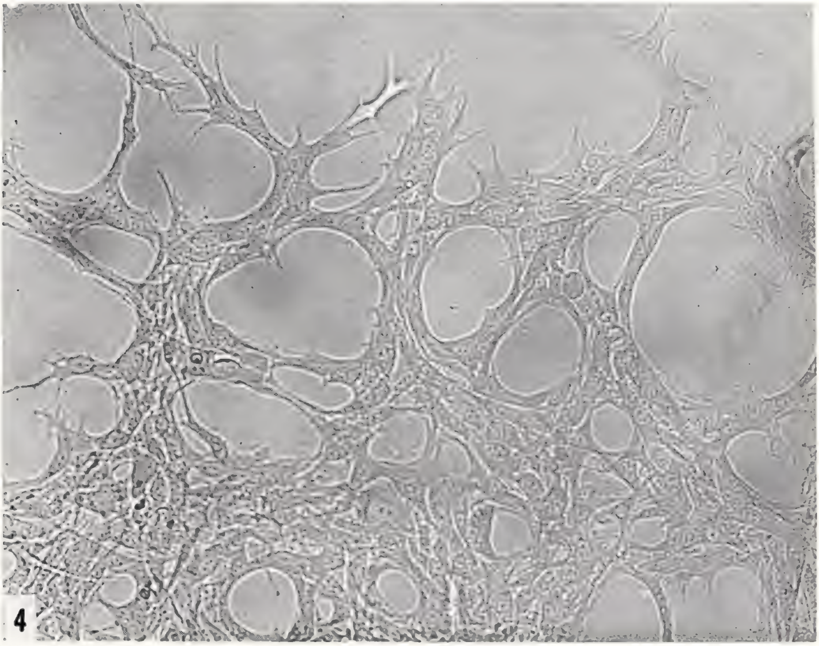
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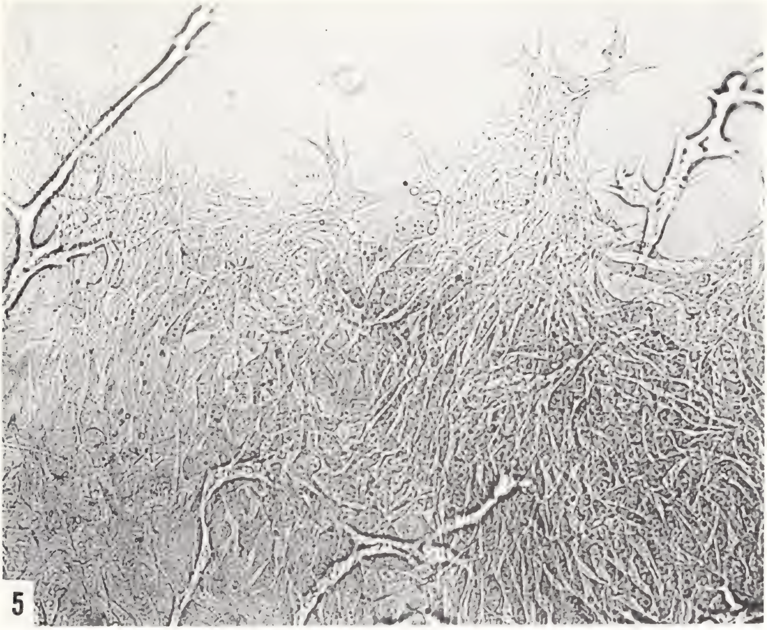
PLATE 22

FIGURE 5.—Further progression of type of change seen in figure 4. Cells have become more sheetlike and are laterally coherent into a continuous sheet. *Bottom* is toward *center* of explant. × 200

FIGURE 6.—Production of relatively tremendous cells epithelial-like in appearance, from cells of same general strain shown in figures 1 through 5, as described in text. × 200



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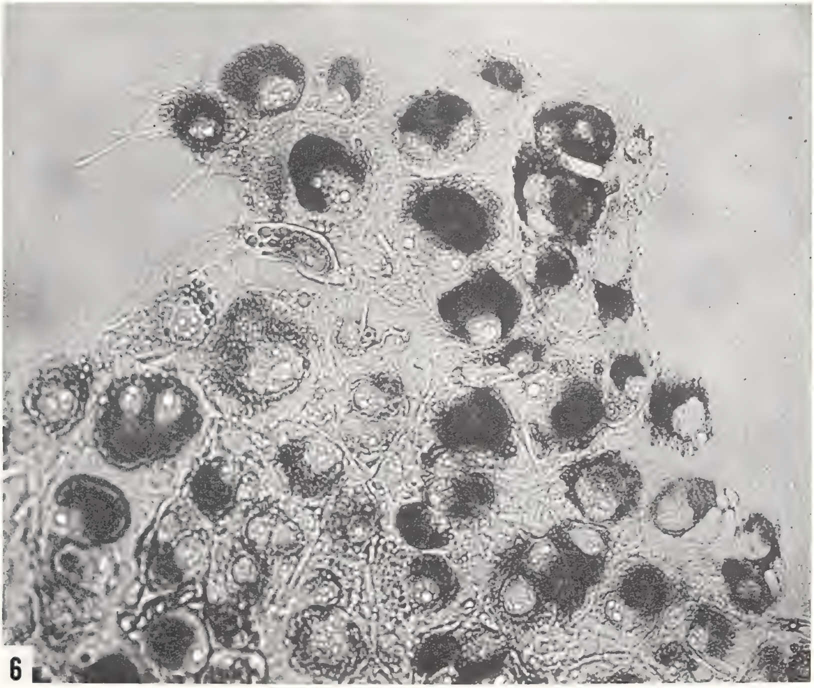

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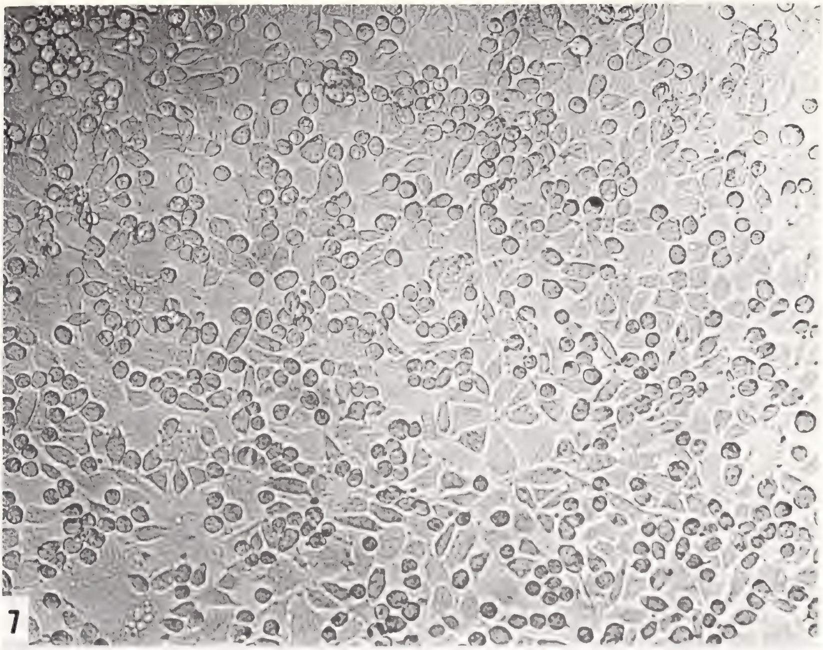
Plate 23

Figure 7.—NCTC clone 929 fibroblasts, growing as a dense monolayer on a glass substrate. *Note* appearance of elongate, sheetlike, and more spherical forms at this stage of cell population crowding. × 200

Figure 8.—Cells as in figure 7, except at greater cell population crowding. Cells are nearly all approximately spherical. *Black* areas are due to cell necrosis. × 200



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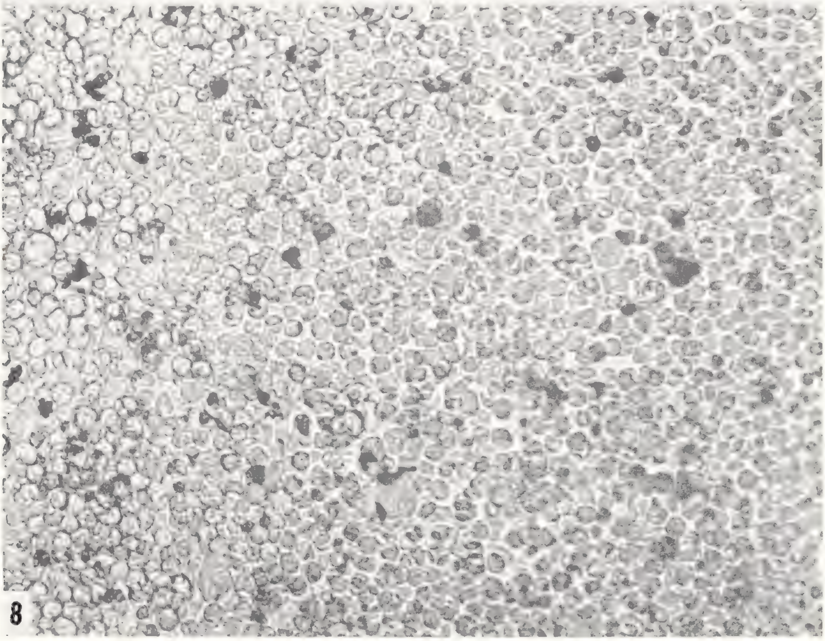

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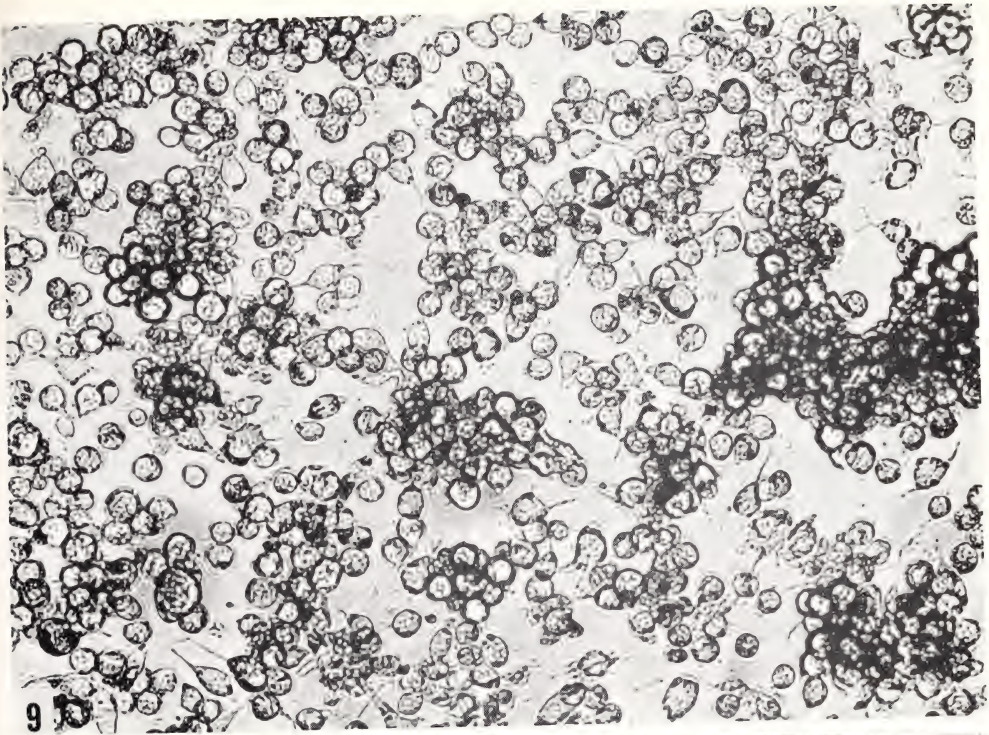
PLATE 24

FIGURE 9.—NCTC clone 929 fibroblasts on glass substrate showing various cell forms and tendency to clump. Medium is 20 percent chick-embryo extract, 30 percent horse serum, in Earle's saline. × 200

FIGURE 10.—Strain NCTC 2071, derived from clone 929 and maintained in protein-free medium NCTC 109. Cell morphology is extremely different from that shown in figure 9. *Note* absence of cell cohesion with consequent absence of clumping. × 200



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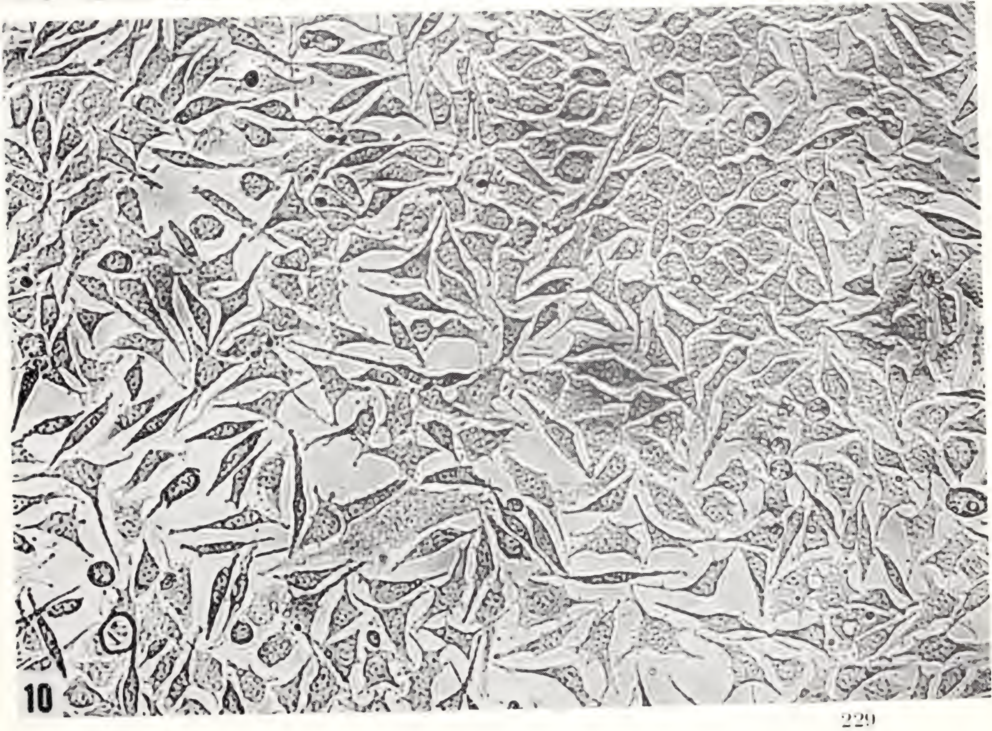

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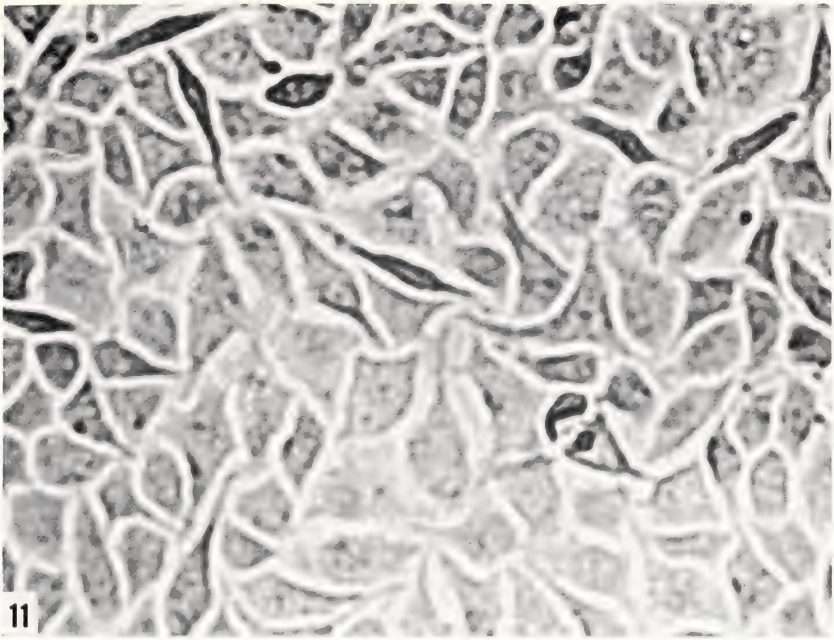
EARLE

Plate 25

Figure 11.—High magnification of dense monolayer sheet of cell type shown in figure 10, glass substrate. Even with this high population density *note* lateral separation of cells from each other. × 400

Figure 12.—Human adult skin epithelium in a relatively young culture started from a single cell in capillary tube at *lower left*. *Note* close cohesion of cells of sheet. × 200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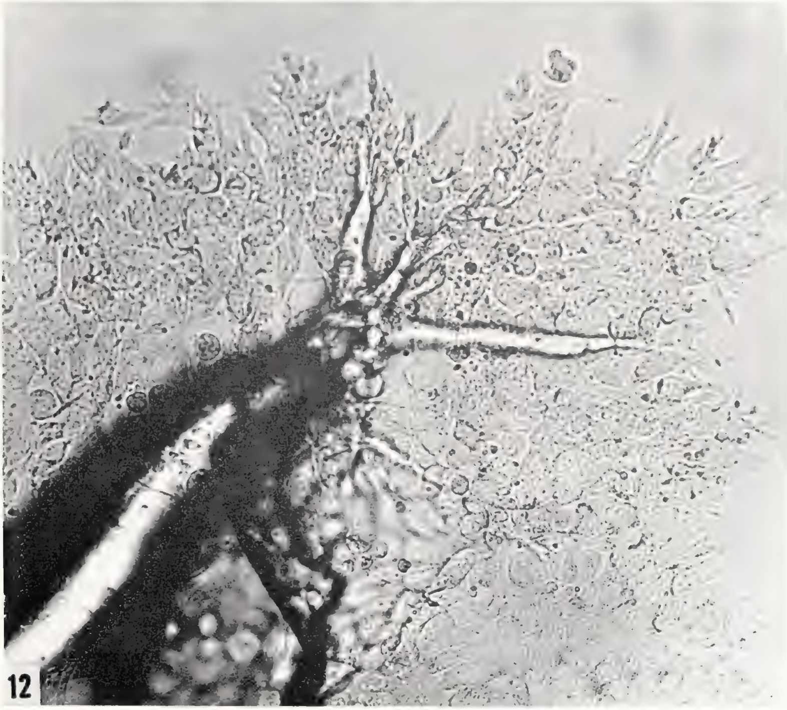
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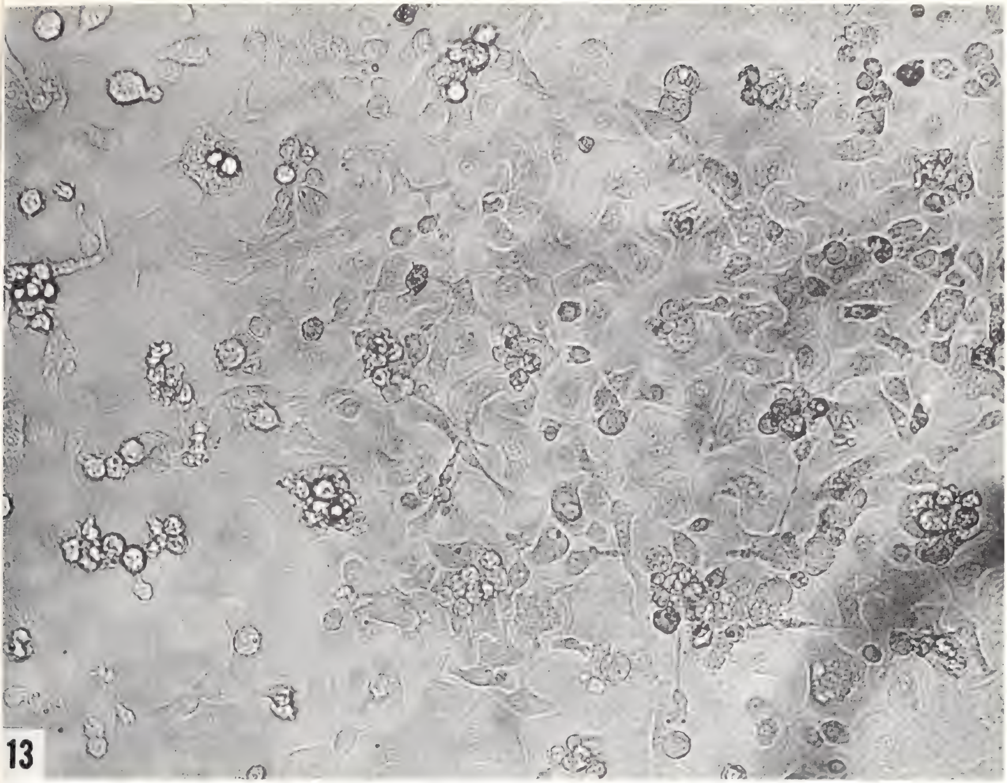
PLATE 26

FIGURE 13.—Cells of liver epithelium, strain NCTC 721 growing under cellophane after 6 months in culture. *Note* predominance of typical sheetlike cells adherent to glass substrate. Rounded and clumped cells are often those that have floated loose. × 200

FIGURE 14.—Cells of above strain on glass substrate after more than 1 year in culture show progression of change in morphology. × 200



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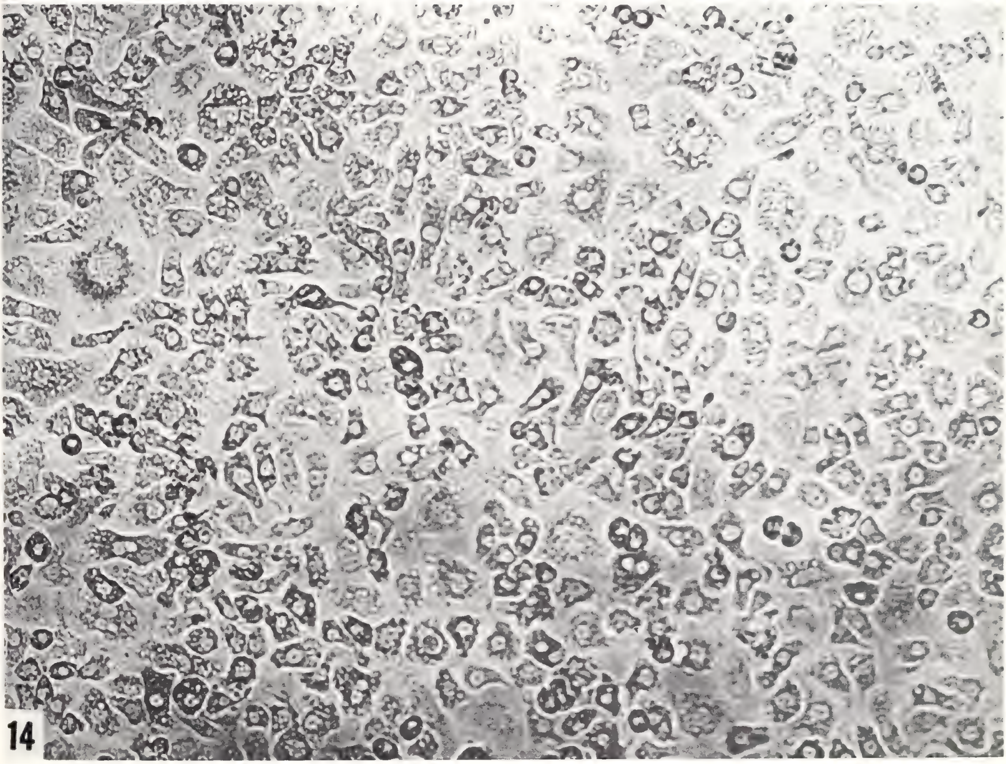

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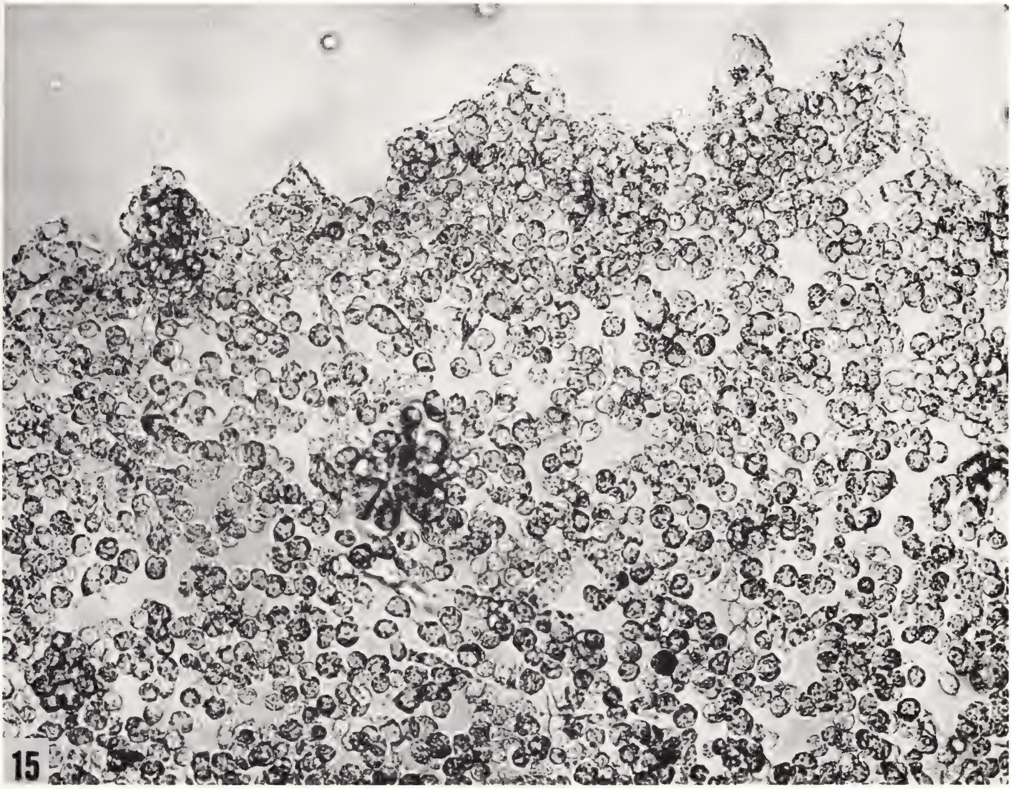
Plate 27

Figure 15.—Later stage in morphologic characteristics of this strain after more than 8 years in culture. At this time strain had been cloned for more than 4 years and was designated NCTC 1469. × 200



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EARLE

DISCUSSION

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No manuscript was submitted.

Session IV

Cells, Viruses, and Cancer—A New Microbiology

Chairman: JOSEPH L. MELNICK, *Ph.D., Baylor University College of Medicine, Houston, Texas*

Enterovirus Ribonucleic Acid in Tissue Culture [1,2]

KATHERINE SPRUNT, *M.D., and* HATTIE E. ALEXANDER, *M.D., Babies Hospital (Presbyterian Hospital), and the Department of Pediatrics, College of Physicians and Surgeons, Columbia University, New York, New York*

FREE INFECTIOUS ribonucleic acid (RNA) has now been derived from a number of animal viruses and studied with considerable interest because it supplies a potentially useful tool for improving our understanding of the function of RNA, infectious and otherwise, in animal cells. Certain requirements for RNA infectivity are common to almost all tissue-culture systems studied so far and are, therefore, of particular interest to cell biologists and virologists.

In this paper our experience with the requirements for infectivity in tissue culture demonstrated by RNA derived from various members of the enterovirus group is presented. The RNA is freed of its protein coat by a modification of the method of Gierer and Schramm (*1*), which consists of shaking suspensions of whole virus (crude or purified) with phenol. The phenol is removed by centrifugation and ether extraction; the ether, in turn, is removed by bubbling with nitrogen. The resulting solution contains a quantity of RNA of which, according to infectivity studies, only a small part is infectious. This infectious portion is believed to be RNA in that it is inactivated by purified ribonuclease (RNase) in very small quantities—less than $10^{-5}\,\gamma$ per ml under some circumstances (*2*). Moreover, specific antibody, which inhibits the infectivity of intact virus, does not influence the infectivity of the nucleic acid. RNA will penetrate and stimulate to production of intact virus any cells that the virus enters and many cells not normally susceptible to whole virus (*3*, *4*). As recipient host, we use the Fernandes passage line of amnion cells (*5*), HeLa cells originally derived from a clonal population selected for increased sensitivity to polioviruses (*6*), and HEp-2 cells. Routinely, 3-day-old monolayers in 60 mm petri dishes are freed of growth medium containing serum by being washed with isotonic salt solution and inoculated with 0.1 ml quantities of RNA. After 30 minutes to allow for adsorption of the RNA, the plates are layered with nutrient medium in agar. Infectivity is measured by the number of infectious centers (plaques) produced. Under appropriate environmental conditions, which will be discussed subsequently, infectivity of an RNA suspension is

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[1] Presented at the Syverton Memorial Symposium on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

[2] Aided by a grant from The National Foundation.

inversely proportional to dilution, which indicates that a single "RNA unit" is infectious.

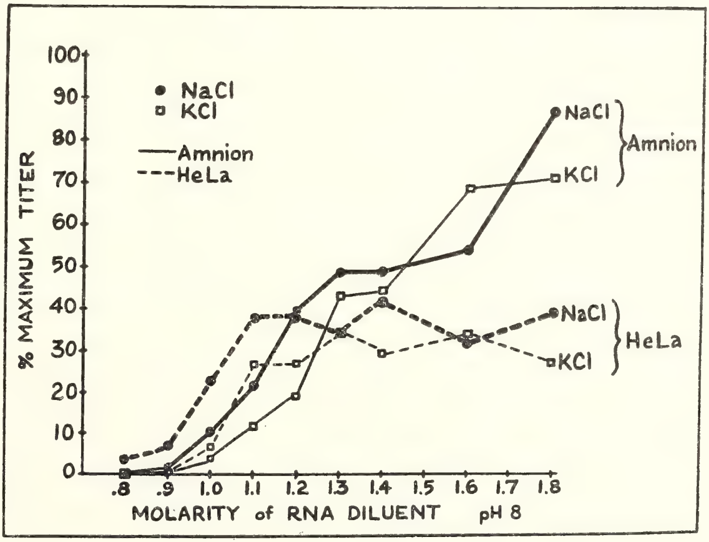
The appropriate environmental conditions that provide the highest degree of infectivity are unusual. Of prime importance is the requirement of hypertonic salt concentration for infectivity in tissue culture. High-titer infectious RNA, diluted in water, rapidly loses its infectivity, but, when diluted further into a diluent containing enough salt, infectivity is immediately and quantitatively regained (*7*). Infectivity is first noted in our system in solutions containing about 0.7 M NaCl and increases with most cell lines until the limit of cell tolerance for salt is reached, about 1.8 to 2 M. It is evident that shifts of such degree from the hypo- or isotonic ranges to hypertonic ranges will exert marked effects on the RNA molecule itself and on the host cells. It could be, however, that an additional extraneous factor, such as host cell RNase, is playing an important role.

It is established that RNA is more sensitive to the action of RNase in an environment of low ionic strength (*8*). Moreover, infectious RNA in isotonic salt solutions is permanently inactivated when exposed to host cells; the same RNA in hypertonic salt solution is not. We have shown that there is an inhibitor, probably RNase, on the surface of monolayers that inactivates RNA in isotonic but not in hypertonic diluent (*2*). This "RNase" can be inactivated by various substances, such as yeast nucleic acid. If monolayers are pretreated with yeast nucleic acid in isotonic salt solution and then inoculated with RNA in isotonic solution, the latter can be removed and shown to be biologically active by an increase in the salt concentration to the hypertonic range. A substance acting like RNase was demonstrated in the extracellular fluids of amnion, HeLa, and HEp-2 cell monolayers and may be a general cell characteristic. Although this RNase-like substance is present on monolayers washed with isotonic salt solutions, it is probably not solely responsible for the lack of infectivity of "isotonic" RNA. Relatively little, if any, "isotonic" RNA seems to be adsorbed, in 10 minutes at least, by cells washed with isotonic salt solution (*2*). Hence, the hypertonic salt solution seems to do more than just decrease the activity of RNase. The important effect may be on the RNA molecule itself, on the host cell, or on both.

Doty has proposed that RNA molecules consist of "single polynucleotide chains" which contract on themselves in hairpin-like helices in moderate salt concentration and which "expand greatly" to unkinked strands in lower salt concentrations (*9*). It is possible that a long, expanded molecule cannot physically be engulfed by a cell and that a more contracted one can be. All that is known now, however, is that "isotonic" RNA is not infectious when exposed to cells in an isotonic environment.

Is the fact that isotonic RNA is not infectious for cells in an isotonic environment a reflection of the physical state of the molecule, or of the competency, or susceptibility, of the recipient population? This question is difficult to answer because the salt effect is so rapid. It is true that cells previously washed in hypertonic solutions can be infected by RNA inoculated in isotonic diluent (*2, 10*). However, almost invariably under such circumstances, the plaques caused by infected cells are characteristically distributed in the gutter of the plate where incompletely removed high salt wash would be expected to accumulate. Occasionally another characteristic distribution pattern is found consisting of a half-moon design of infected cells extending from the middle of the plate to the gutter and corresponding to the area where salt might be expected to have adhered to the cells as it drained across them. We have not noted these characteristic distribution patterns under other circumstances and think they are due to infection by RNA in residual hypertonic salt. We have tried many times to condition cells into a state of competency before inoculation by incubating them in high salt concentrations. Immediately before inoculation, the salt was removed as completely as possible, and the cells were flushed with isotonic salt solution. Isotonic RNA was then added. The whole procedure, from removal of high salt concentration from the monolayers to inoculation, took only about half a minute. Under such circumstances, no plaques have formed. Unfortunately, since the salt effect is so rapid, the procedure proves only that pretreatment of cells with salt does not render them susceptible to isotonic RNA a few seconds thereafter. The molecule-versus-the-cell debate is still open, though most probably the changes occurring in both in hypertonic salt are of significant importance.

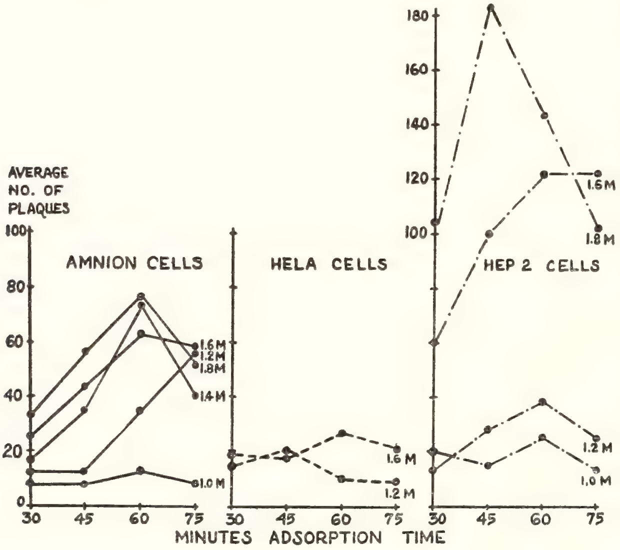
For whatever reasons, however, hypertonic environments are required during the adsorption period to demonstrate infectivity. Optimum conditions, with relation to salt concentration, vary to some extent, depending on a number of factors known and unknown, but primarily on the choice and physiologic state of the recipient cell. Text-figure 1 shows



TEXT-FIGURE 1.—Effect of NaCl and KCl concentration of diluent on titer of RNA in amnion and HeLa cells. Points represent average of values from 5 experiments.

the results of experiments in which both amnion and HeLa cells were inoculated with the same RNA preparation suspended in increasing quantities of NaCl and KCl. It is evident that HeLa cells are slightly more susceptible than amnion cells to RNA at salt concentrations below 1 M, but there is little titer enhancement with greater salt concentrations. On the other hand, amnion cell sensitivity to RNA infection is roughly proportional to the salt concentration until the limit of cell tolerance for salt is reached at 1.8 to 2 M. The response of HEp-2 cells is similar to amnion cells with a higher degree of RNA infectivity throughout the range of salt concentrations.

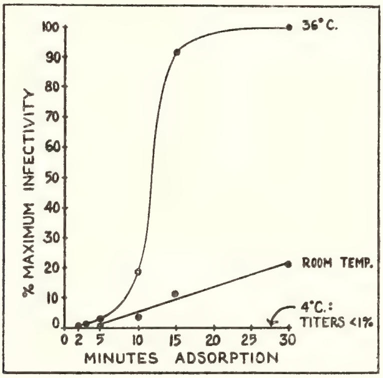
In these experiments a standard 30-minute adsorption time was used. Text-figure 2 shows that, if this time interval is extended, a similar difference between cell lines is demonstrated. In amnion and HEp-2 cells there is a significant increase in infectivity of RNA with time between 30 and 60 minutes after seeding and with increases in salt concentration above 1 or 1.2 M. In HeLa cells no increase in infectivity occurs with adsorption times over 30 minutes, and there is no significant enhancement with salt concentration over 1 M.



TEXT-FIGURE 2.—Effect of time of adsorption and salt concentration of diluent on titer of RNA in three cell lines.

There are other factors of known importance in enhancing RNA infectivity. The most notable of these are *p*H and sucrose (*11*, *12*), both of which cause increase in infectivity as they themselves increase in concentration to the point of cell toxicity. In our monolayer test system, however, the infectivity-enhancement effects of *p*H and sucrose are demonstrable only when added to diluents containing hypertonic quantities of salt. We have been unable to demonstrate infectivity of RNA in 0.14 M NaCl even though up to 40 percent sucrose was added. Colter, who uses a suspended cell test system and RNA of different origin, is able to obtain infectivity in high titers with diluents containing only about 17 percent sucrose in isotonic salt (*12*). This suggests that the "salt effect" is largely osmolar on the host cell. It must not be quite this simple, however, since sucrose will not substitute for salt in our system.

In the situations described so far, the diluent, cell, or cell environment has been the variable. As would be expected, infectivity of RNA in constant diluent is variable, depending on the physiological state of the host cell. Text-figure 3 shows that cell environmental temperature has a marked effect on RNA infectivity titer. Colter (*13*), Koch (*14*), and we have shown that infectivity decreases to a very low point at 4° C, increases at room temperature, and is much greater at 36°. An active process on the part of the cell seems to be involved in production of infectivity starting at the level of RNA adsorption (*15*). Table 1 indicates that monolayers of amnion and HEp-2 cells grown for 1 day are clearly less susceptible to RNA infectivity than cell layers incubated 2 to 4 days prior to inoculation. This suggests that cells in the logarithmic stage of growth are more competent than those in the lag phase (*16*). The nutritional state of host cells is also important. Table 2 shows the results of some experiments in which we tried the magnesium sulphate-calf serum system recommended by Syverton's group (*17*). They feed their monolayers with 20 percent calf serum and suspend their RNA in 2 M $MgSO_4$, a combination which gives them their best titers. The procedures in the



TEXT-FIGURE 3.—Effect of temperature on rate of adsorption on RNA.

TABLE 1.—Effect of age of monolayer on titer of RNA

Age of monolayers (days)	Amnion cells experiment No. 1*	2	3	HEp-2 cells experiment No. 1*	2	3
1	4†	5	7	14	23	25
2	10	31	18	36	62	62
3	21	53	30	24	58	69
4	12	15	26	31	61	88

*Each experiment done simultaneously in the 2 cell lines.

†Average number of plaques on 4 monolayers.

experiments shown in text-figure 3 were unusual in that the cells were refed 24 hours before inoculation with the feeding solutions indicated. RNA was suspended in 2 M $MgSO_4$ or 1.6 M NaCl. It is evident that cell feeding solution had little effect on infectivity when RNA was suspended in NaCl, but there was a marked effect when RNA was suspended in $MgSO_4$. It may be that $MgSO_4$ is somewhat more damaging to the cells and that calf serum maintains them in a healthier and, hence, more susceptible state.

Adequate attention to the physiological state of the cell is exceedingly important when one is trying to do reproducible quantitative work with RNA. Most of the infectivity-enhancing factors have maximal activity as they approach the point at which they produce cell damage, and, at the point of early cell damage, ability of the monolayer to support growth of virus is very irregular. Working too close to the limit of cell tolerance is responsible, we think, for some of the irregularities encountered in tests for reproducibility of experimental results (*10*). For instance, we have noted that when 0.2 ml of RNA in 1.6 M NaCl was inadvertently used as inoculum instead of 0.1 ml, we obtained lower titers than expected. This led us to re-examine the relation of dilution factor to infectivity with the results shown in table 3. The actual plaque count obtained is indicated in each block, with the expected count as determined from the plates with the highest and presumably most accurate plaque counts in parentheses. As shown in the upper section of the table, more RNA achieved by inoculums of larger volume apparently produced fewer plaques. On the hypothesis that this result might be due to injury of the cells by the unusual amount of salt incorporated in the agar overlay at the end of the adsorption period, similarly inoculated plates were rinsed with isotonic salt solution just before the agar layer was added. The bottom section of the table indicates that the results then correlated well with the expected ones. The additional 0.1 ml of 1.6 M NaCl diluted in 4.0 ml of isotonic overlay is sufficient to produce irregular results.

In summary, a hypertonic RNA-cell environment is essential for infectivity of RNA in tissue culture. Many secondary factors affect the degree of infectivity of a hypertonic preparation, such as choice of host cell, RNA adsorption time, *p*H temperature, and age of cells. The highest

TABLE 2.—Effect of environment of 3 cell lines during growth on susceptibility to RNA diluted in 1.6 M NaCl and 2 M $MgSO_4$

Growth medium: Monolayers refed 24 hours before inoculation with Eagle's medium + 20%:	Plaque count* produced by RNA					
	Amnion cells		HeLa cells		HEp-2 cells	
	NaCl	$MgSO_4$	NaCl	$MgSO_4$	NaCl	$MgSO_4$
Calf serum	12	23	33	31	24	27
Horse serum	18	12	29	22	29	14
Human serum	12	13	28	12	24	11
Not refed	11	4	23	4	26	8

*Plaque count averaged from 6 experiments; 3 or 4 plates per lot per experiment.

TABLE 3.—Relationship between plaque count and dilution of RNA

RNA dilution in 1.6 M NaCl	Average No. plaques produced by RNA in following size inoculum:			
	0.1 ml	0.2 ml	0.3 ml	
1×10^{-3}	12 (11)*	14 (24)†	7 (36)†	No wash before agar layering, 3 plates/dilution
2×10^{-3}	31 (22)			
3×10^{-3}	33 (33)			
1×10^{-3}	10 (13)	13 (20)†	29 (30)†	Isotonic wash before agar layering, 6 plates/dilution
2×10^{-3}	25 (26)			
3×10^{-3}	28 (39)			
4×10^{-3}	51 (52)			
5×10^{-3}	67 (65)			

*Value in parentheses = expected count as calculated from count produced by most concentrated sample.
†Calculated from most dilute sample.

infectivity titers will be obtained, however, only when these factors are carefully controlled so that the physiological state of the recipient cell is not compromised beyond recovery.

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DISCUSSION

John S. Colter, *Wistar Institute, Philadelphia, Pennsylvania*

Dr. Sprunt, in her presentation, has raised several questions to which, I believe, I can provide at least partial answers.

Dr. Sprunt has indicated that my colleagues and I use a suspended cell system for the assay of infectious viral ribonucleic acid (RNA) preparations. Very briefly, our technique is as follows: Washed cells, usually 1 to 2 × 10^6 cells, are suspended in 1 ml of the viral RNA, dissolved in whatever medium we wish to examine. The mixture is incubated at the desired temperature for the desired time, after which it is diluted tenfold with tissue-culture medium, a manipulation which immediately and completely abolishes any further cell-RNA interaction. The number of infectious centers produced is then measured by titrating the suspension of treated cells on preformed monolayers of the appropriate cell type.

I do not wish to dwell at length on the advantages of the suspended cell system. However, some of the more obvious ones might be mentioned. The conditions under which cells and RNA interact can be precisely defined. By the avoidance of exposure of the indicator monolayer to solutions of elevated osmolarity, the cell degeneration which often accompanies this manipulation is avoided. As a result the plaques are always well defined and readily counted. By flooding the monolayer with a suspension of cells, we obtain a uniform distribution of plaques. Finally, the ease with which the system can be manipulated, coupled with the fact that dilution of the cell-RNA mixture with culture medium completely stops further interaction, has made it possible to define, with considerable precision, the kinetics of the interaction.

In regard to the optimal conditions for cell-RNA interaction, we have examined the effects of osmolarity on the interaction of Mengo RNA and strain L cells and of polio RNA and HeLa cells using two series of diluents: 1) solutions of NaCl buffered at *p*H 7.3 with 0.02 M phosphate buffer (xM PBS) and 2) solutions of sucrose in phosphate-buffered physiological saline (xM Suc/PBS). In both systems, and with both series of diluents, the number of infectious centers produced increased with increasing tonicity up to a sharp maximum, beyond which the number of infectious centers produced decreased sharply. In the Mengo RNA-L cell system, the optima are seen at 0.7 M Suc/PBS and 0.64 M PBS, with the optimal sucrose solution 1.5 to 2.0 times as efficient as the optimal saline solution in promoting infectious center formation. In the polio RNA-HeLa cell system, the optima are found at 0.98 M Suc/PBS and 0.90 M PBS, with 6 to 7 times as many infectious centers produced in the optimal saline as in the optimal sucrose solution.

These observations make it clear that the optimal conditions for cell-RNA interaction are a function of the cell type used. In both the HeLa and strain L cell systems, the same phenomenon with respect to osmotic pressure is observed. With each cell type, the optimal osmotic pressure for the formation of infectious centers is the same in both the saline and sucrose series. The fact that it is higher for HeLa (5.7 × isotonic) than for strain L (4.1 × isotonic) seems to be due to the greater resistance of the former cell to this type of stress. The finding of an optimal osmotic pressure in both diluents suggests strongly that the withdrawal of water from the cell, which thus creates a hypertonic intracellular environment, is the important factor in increasing the efficiency of infection with RNA. In our minds, the "RNA molecule versus the cell debate" is resolved. The effect of hypertonic solutions is on the cell.

In both the HeLa and strain L cell systems, the number of infectious centers formed in cell-RNA mixtures is directly proportional to the concentration of RNA and to the number of cells used, and in both, the interaction is strongly temperature-dependent with an apparent energy of activation of approximately 34,000 calories per mole.

The rate of formation of infectious centers at 37° C in HeLa cell-polio RNA (in 0.90 M PBS) and strain L cell-Mengo RNA (in 0.70 M Suc/PBS) mixtures is linear with respect to time for 8 and 15 minutes, respectively. When incubations are continued for longer periods, there is a precipitous drop in the number of infectious centers. We have demonstrated that the reason for the sharp breaks in the curves relating the number of infectious centers to time of incubation at 37° C is due to the rapid loss of cell viability (as measured by the ability of the cells to support viral replication) at about these times.

Studies of the uptake of RNA-P^{32} by strain L and HeLa cells have provided an explanation for the low infectivity of viral RNA preparations compared with those of the corresponding viral suspensions. In these studies we used labeled, high molecular weight cellular RNA, isolated by the phenol method and purified by repeated precipitation from 1 M NaCl. Our conclusions arising from this work are based on the assumption that the cells cannot differentiate—insofar as uptake is concerned—between cellular and viral RNA.

With both HeLa and strain L cells, the amount of RNA-P^{32} absorbed by the cells from physiological saline and from the optimal sucrose solutions was precisely the same. The rates at which both cell types absorbed RNA from their optimal saline solutions increased with time of incubation. However, the increased rate of uptake was directly correlated with loss of viability of the cells and was clearly due to binding of the nucleic acid by damaged cells. We found that 10^7 L cells absorbed only 0.054 percent of the RNA present in 0.5 ml of solution in 15 minutes at 37° C. Thus 10^6 cells in 1 ml RNA solution, standard assay conditions, would absorb only $0.1 \times 0.5 \times 0.054 = 0.0027$ percent of the total RNA present. Therefore, to correct for this nonabsorption, the observed titers of Mengo RNA preparations should be multiplied by 1/0.000027 or approximately 4×10^4. A similar correction factor should be applied to the observed titers of polio RNA, since 10^7 HeLa cells were found to absorb only 0.034 percent of the total RNA present in 0.5 ml of solution in 8 minutes at 37° C. In both cases, the correction factor is large enough to bring the titers of the RNA preparations in line with those of the corresponding virus suspension.

To summarize, we believe that:

1) The uptake of viral RNA by cells is a case of concentration-dependent diffusion across the cell membrane.

2) The effect of solutions of high osmolarity in increasing the efficiency of infection with viral RNA is on the cell (not on the RNA), and the basic effect is to create a hypertonic intracellular environment by dehydration of the cells. The hypertonic extracellular environment does not stimulate RNA uptake, at least not by cells capable of supporting viral replication, but the hypertonic intracellular environment protects the RNA from inactivation after it is absorbed.

3) The low infectivity of viral RNA preparations can be completely accounted for by the low permeability of the cell membrane to this macromolecule. All current methods for the assay of viral RNA merely measure the initial rate of reaction between nucleic acid and cell. Carefully isolated viral RNA preparations contain at least as many potentially infectious units as do the corresponding virus suspensions.

Clonal Variation and Interaction of Cells With Viruses [1, 2]

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A CONSTRUCTIVE discussion of "Clonal Variation and Interaction of Cells With Viruses" can be attempted only if there is a clear understanding of the illusive and ever-changing vocabulary of cell culture and virus research. For clarity a number of the terms used are defined in table 1. To provide illustrative data, related to the conceptual and technological problems associated with an investigation of clonal variation and virus susceptibility, representative experimental findings from our studies will be presented so that they can be used as a willing target for constructive criticism.

For convenience, reference will be made to the test system summarized in table 2. The 4 strains of cells, HeLa-S3, HeLa-Gey, Chang-liver, and Maben, represent a spectrum of wild-cell types differentially susceptible to cytopathic infection by a submoderate (noncytopathic) variant of type 2 poliovirus, MEF_1 strain (*2*). The findings listed in table 2 show

TABLE 1.—Definitions of cell culture and virus terminology*

Term	Definition
Virulent mammalian viruses	Cause rapid destruction of cells; ordinarily do not cause latent infection of cell cultures, *e.g.*, common cytopathic enteroviruses.
Submoderate mammalian viruses (*1*)	Give rise to latent infection of cell cultures because of moderate cytopathicity and differential susceptibility of cells to infection; individual cells not hereditarily infected, *e.g.*, basophilic, respiratory, or Arbor viruses.
Wild-cell population	Any population of cells that has not been characterized.
Colony of cells	A population of cells (colony) which may result from growth of single or multiple cells.
Clone of cells	A population of cells derived from one parent cell, *as proved* by suitable tests that employ a reasonable number of genetic markers.

*Definitions listed are of the "operational" category; they should not be confused with classical genetic terminology.

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[1] Presented at the Syverton Memorial Symposium on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

[2] These studies were supported jointly by Public Health Service grant E2279-C and United States Army Chemical Corp. grant FD-GR-60-5.

TABLE 2.—Spectrum of interaction between a submoderate variant of type 2 poliovirus and representative cell strains

Cell strain	Serial propagation of: Virus	Serial propagation of: Infected cultures	Cytopathic effect of virus
HeLa-S3*	+	+	−
HeLa-Gey†	+	−	+
Chang-liver‡	+	±	±
Maben§	+	±	±

*Cells and virus propagated simultaneously and serially without overt morphologic evidence of virus infection in medium free of antibody to poliovirus. HeLa strains of diverse origin reacted differently to infection (*2*).

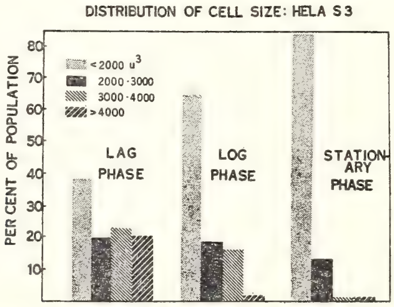
†The HeLa-Gey strain of cells was fully susceptible to rapid destruction by submoderate poliovirus.

‡Minimal partial destruction (±) of the Chang-liver cultures occurred in the initial passages of virus in this cell strain. The wild population of Chang cells selected for virulent (cytopathic) mutants of virus (*2*).

§Significant (±) but incomplete destruction of Maben cells occurred as a result of submoderate poliovirus infection (*2*).

that the virus propagated in each of the 4 cell strains. No cytopathology was evident either in the HeLa-S3 or the Chang-liver strains of cells. Simultaneous serial propagation of both virus and cells occurred regularly only with select strains of HeLa cells (*2*). The additional results were obtained from studies designed to determine the reasons for the differential response of the test cell strains to submoderate poliovirus infection. Thus, it was necessary first to analyze carefully each of the cell populations to understand "clonal variation and virus susceptibility." Concomitantly, it was essential to bear in mind factors of known importance to the latent infection of cells in culture by submoderate viruses (*3*).

The methods applied to the analysis of the 4 prototype cell strains were selected so that they would be both informative and applicable to biochemical and genetic studies of submoderate virus-cell relationships. Accordingly, an assay was made of the distribution of cells of different sizes in the test cell strains. The data (text-fig. 1) for the HeLa-S3 cells are representative of the 4 cell strains. The results established that the ratio of small to large cells increased as a function of the phase of cell growth (*4–6*), though small cells predominated at each portion of the growth curve. The significance of these observations is that the fluctuation in the chemical composition of cell populations as reported in the literature (*7–9*) undoubtedly represents a composite of the simultaneous change in (*a*) cell size, (*b*) chemical composition of individual cells, and



TEXT-FIGURE 1.—Cell size was determined by use of a calibrated ocular micrometer (*10*).

(*c*) distribution of "variants" in wild-cell populations. While these observations have implications in the interpretation of data dealing with the chemical analysis of cells infected by cytopathic mammalian viruses, undoubtedly they are particularly relevant to biochemical and genetic studies of submoderate virus replication in heterogeneous (wild) cell cultures. Moreover, such variations in cell populations can account, in part, for discrepancies in reported values of the chemical composition of primate cells in culture.

To additionally characterize wild-cell populations, and to gain insight into some causes in the phenotypic variation (*11, 12*) of mammalian cells in culture, growth curves were carried out. Representative data (at least 3 replicate experiments/cell strain) are summarized in text-figure 2. The mean generation time of 46 hours observed for the Maben strain of cell contrasted sharply with the 31-hour generation time noted for the other cell populations.

To test whether readily evident differences could be observed in the growth rates of a wild population of cells and a genetically pure line derived therefrom, analyses of the test cell strains were carried out as indicated previously. The relationship between the growth rate of a wild population of HeLa-S3 cells and a clonal derivative is illustrated in text-figure 3. The data show clearly that the growth rate of the genetically pure cell variant was essentially half that of the wild population.

Because host-cell physiology (*13, 14*) is particularly important in governing the susceptibility of cells to submoderate virus infection and may serve as a parameter of phenotypic variation (*15, 16*), experiments were done to assess the extent to which feeding of cells could alter their mean generation time. The selected findings summarized in text-figure 4 reveal the influence of feeding the cell inoculum prior to its use in growth-curve experiments. It is evident that the fed cells had a much higher content of ribonucleic acid (RNA) than those not fed. Similar findings have been reported by others (*17*). Collectively, the data cited may help explain discrepancies in the literature concerning the comparative biochemical properties of either normal or virus-infected cell cultures.

These tedious but essential preliminary experiments were a prerequisite to a more meaningful delineation of some of the factors influencing "clonal variation" and interaction of cells of colonies with submoderate viruses. From the results of the survey experiments (*2, 18*) it was possible to select a spectrum of operational genetic markers for cells in accordance with their appropriateness to our needs. A minimal essential number of these markers (distinctive morphologic features of cells and cell colonies, susceptibility of cell colonies to submoderate virus infection, growth rate, and chemical composition of cells) was used to study the accuracy of conventional[3] cell-plating techniques (*19*) in providing genetically pure cell colonies (clones). These experiments were instituted because an investi-

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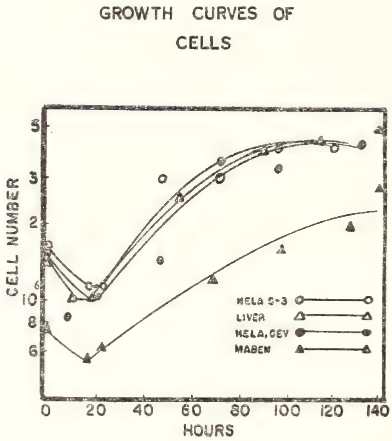
[3] The "Puck technique" (*19*) is often cited by authors as a basis for the isolation of "cell clones" when, in fact, the exact procedure and precautions listed in the reference publication are not followed. Thus, the term "Puck technique" as used in the literature may have quite an obscure meaning.



TEXT-FIGURE 2.—Stationary phase cells were used as inoculum for growth curve vessels. Cultures were not fed or treated in any way during experiments.

gator may not have the option of assuming that a cell colony is genetically pure (*10*, *20*, *21*). This viewpoint, if it has merit, is basic to any laboratory study designed to yield information concerning "mutation rates" of cells (*22*, *23*), analysis of cell karyotype (*24–27*), genetic alteration of mammalian cells by viruses, nucleic acids, etc. Similarly, one must distinguish between experiments designed to characterize the population genetics of cell cultures compared with those devised to test changes in the genotype or phenotype of individual cells or of genetically pure cell populations.

To test the accuracy of conventional cell-plating techniques in obtaining genetically pure cell populations, we used 3 specimens of each of 4 distinctive cell types for each of the 4 cell strains. Each cell strain was plated to give well-isolated colonies which were then picked, genetically "repurified" (*19*) 3 times, and grown out in successive culture transfer levels. At each transfer level, so-called clonal populations were plated to test for genetic purity, *i.e.*, samples of each population were plated to yield from 250 to 300 colonies (*ca.* 0.01 % sample of the parent population). Initial passages appeared to be genetically pure (table 3) because sampling of populations limited adequacy of tests, *i.e.*, when cultures contained only a few "contaminant" cell variants they were not detectable. By continuous growth of cultures, such "contaminants" became abundant enough to detect if a large enough sample of the population was assayed. These difficulties were essentially eliminated when the cell-plating technique and the "terminal dilution technique" were used in combination (*20*). Since the combined techniques usually yielded populations genetically pure for the markers employed, one is led to conclude that the test cells were not inherently unstable genetically.[4] The data cited as representative confirm a common viewpoint (*10*, *20*, *21*): That

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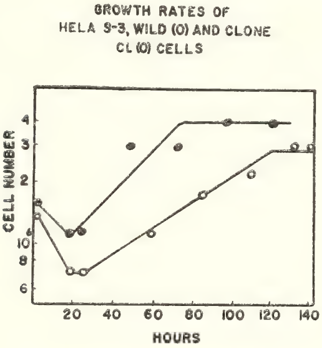
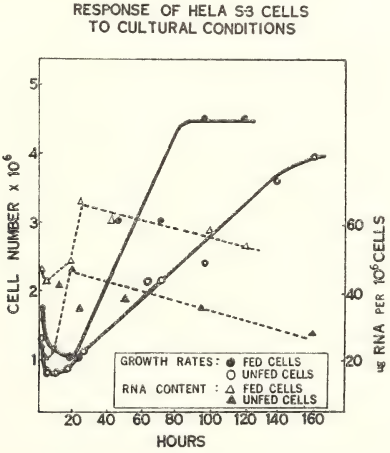
[4] Eleven of the 18 variants studied appear to be genetically stable; the remaining 7 may be very difficult to genetically purify and/or genetically unstable.



Text-figure 3.—Growth rate of a clonal population (*lower curve*) was essentially half that of the wild population (*upper curve*).

genetic purity does not automatically result from plating of cells. Thus, "clonal variation" resulting from genetic impurity of cell populations can and must be distinguished from "clonal variation" of genetically pure cell strains attributable to genetic and/or phenotypic changes of individual cells and their derivative populations. The experimental results previously noted also reveal differences in the ease with which genetically pure cell lines are obtained. Some cell variants, which were small, cohesive, and difficult to make monodisperse, were very difficult to purify.

The morphologic characteristics of individual cells, when correlated with their colony morphology, served as a convenient marker for the provisional classification of cells in the populations tested (fig. 1). Thus, all colonies in the test cell strains were divisible into 2 basic groups (*18,*



Text-figure 4.—The data summarized in this figure reveal how phenotypic variation can occur in cell strains in response to culture conditions or techniques used for propagation of cell populations. *Uppermost solid curve* shows cells fed prior to use for growth-curve studies and exhibit a mean generation time of 24 hours. *Lower solid curve* shows unfed cells, with a generation time of 56 hours.

TABLE 3.—Efficiency of cell-plating technique in yielding genetically pure cell colonies (clones)

Cell strain	Colony type of cells	Genetic repurification of colony type	Serial transfer level of "recloned" population	Genetic purity of "recloned" population
HeLa-S3	Compact-large*	Third	0	"Pure"
			1	Pure
			2	Pure
			3	Impure
	Diffuse-large†	Third	1	"Pure"
			2	Pure
			3	Pure
			4	Pure
			5	Pure
			6	Impure

*Made up of round, tightly packed, epithelioid cells (*see* fig. 1).

†Comprised of fibroblastic cells diffusely distributed in translucent mature colonies (*see* fig. 1).

28), compact or diffuse. Compact colonies were made up of cells tightly packed together, epithelioid in shape, very cohesive, difficult to trypsinize and to keep in suspension; the colony itself was multilayered and opaque. Diffuse colonies were comprised of fibroblastic cells, easy to trypsinize and maintain in suspension; mature colonies contained many giant cells and were either translucent or essentially transparent. Each of these basic colonial types was subdivided into colonies designated "large" or "small." Although the classification was applicable to each cell strain, the colonies of each wild-cell population displayed their own distinguishable features. Finer subclassification of variants in cell populations was possible, but data are not currently available to substantiate fully the degree to which such subdivision can be reasonably extended. Obviously, such further subclassification will be influenced by the range and reliability of the genetic markers employed.

The experience gained in the identification of the cell variants described clearly showed that morphologic criteria alone did not permit unequivocal recognition of the colony cell type. Sensitivity of cells of colonies to cytopathic infection by submoderate viruses was the most convenient and sensitive supportive genetic marker. The results tabulated in table 4 correlate colony designation of cell type with susceptibility to submoderate poliovirus infection. The following facts are particularly noteworthy: (*a*) In most of the wild-cell populations, except the HeLa-Gey strain, more than one resistant variant was detectable. (*b*) The reaction of the colony cell type to infection tended to be distinctive for each cell strain though the compact-large (CL) and diffuse-large (DL) variants reacted similarly. (*c*) All the designated test colonies were fully susceptible to infection by virulent type 2 poliovirus, MEF_1 strain. Thus, it seems reasonable to assume that if a virus is used to "purify" a cell strain, particularly if the virus preparation is not genetically pure, the uncomfortable possibility exists that the derived population may be genetically impure for that marker and probably for other markers as well.

The data listed in table 4 are classified as "provisional" because the dose effect (*29*) of submoderate poliovirus on cells (multiplicity of infection) has not been adequately studied.

After the initial analysis of the cell strains was completed, it was possible to study the modal distribution of cell variants in the 4 cell strains tested. Log phase cell populations were plated to yield from 30 to 50 colonies per culture vessel and, after incubation at 37° C for 8 to 12 days, the colonies were classified and enumerated. The data in table 5 reveal that approximately 80 percent of the population of each cell strain was made up of cells giving rise to diffuse-type colonies and that the large and small diffuse-type colonies were present in a ratio of 1:1. Generally, cells that gave rise to compact-type colonies made up 20 percent of the population; the ratio of large to small type compact colonies was particularly distinctive for the Maben strain of cells. In these studies the data were compiled from 4 to 5 experiments for each cell strain, and for each strain a total of from 3,000 to 4,000 colonies was counted and classified. The type of information obtained made it possible to arrive at a standardization of wild-cell populations. Moreover, it permitted a standardization of growth media to yield the same modal distribution of cells possessing equivalent growth rates and chemical composition.

To illustrate how knowledge of the modal distribution of variants in wild-cell populations can be used to characterize the "variation" of cell populations in response to environmental and nutritional conditions, wild populations of test cells grown in human serum and Eagle's medium were

TABLE 4.—Differential susceptibility of cell colonies to infection by submoderate poliovirus

Cell strain	Colony type	Morphologic response of cells to infection by submoderate poliovirus*
	CL†	R
HeLa-S3	CS	R
	DL	S
	DS	S
	CL	R
HeLa-Gey	CS	S
	DL	S
	DS	S
	CL	R
Chang-liver	CS	R
	DL	R
	DS	S
	CL	R
Maben	CS	S
	DL	S
	DS	S

*One hundred infective doses of virus were added to vessels containing colonies to obtain a provisional assay of the relative capacity of cell colonies to resist (R) or undergo cytopathic destruction (S).

†CL = compact-large; CS = compact-small; DL = diffuse-large; and DS = diffuse-small.

TABLE 5.—Modal distribution of variants in log phase cultures of wild-cell populations

	Percent of colony cell type in wild-cell populations*				
Cell strain	Compact-large	Compact-small	Diffuse-large	Diffuse-small	Percent variation
HeLa-S3	16	6	39	41	5–10
HeLa-Gey	12	6	43	40	5–10
Chang-liver	23	3	41	32	5–10
Maben	6	14	37	42	5–10

* Data for each cell strain are the composite results from 4 to 5 replicate experiments; from 3,000 to 4,000 colonies were counted for each cell strain.

adapted separately to growth in Eagle's medium supplemented with either horse or calf serum, respectively. A 5 percent increase in respective heterologous serums was used at each adaptation level until a final concentration of 20 percent was attained; simultaneously, the concentration of human serum was appropriately reduced until a final concentration of zero was reached. Three passages of cells were made at each serum increment level, and the distribution of colony variants determined by the technique of plating as previously noted. To facilitate work, cell colonies developing in test serums were enumerated after Wright's stain was applied (*10*). Identification of colonies was made macroscopically. Small and large colony variants were not distinguished, *i.e.*, all were classified arbitrarily as either compact or diffuse. Thus, the results obtained should not be regarded as definitive as those listed in table 5. The results of these studies (table 6) revealed that a marked selection occurred for the compact colonial cell type at the 20 percent level of heterologous serum, but total suppression of a given cell type did not result. These findings suggest that a sharp distinction can and should be made between a change in the modal distribution of cells in a wild population and variation (modulation) of individual cells.

Separate experiments were carried out in which genetically pure cell lines derived from wild-cell populations were propagated in heterologous serums without preliminary adaptation. Under these experimental conditions the variants tested retained their characteristic morphologic features (table 7).[5]

TABLE 6.—Selective effect of calf or horse serum on wild-cell populations

	Percent distribution of colony variants in Eagle's medium supplemented with 20 percent serum*					
	Human		Calf		Horse	
Cell strain	Compact	Diffuse	Compact	Diffuse	Compact	Diffuse
HeLa-S3	61	39	98	2	96	4
HeLa-Gey	61	39	95	5	86	14
Chang-liver	58	42	71	29	87	13
Maben	57	43	94	6	81	19

*The data reflect the change in the distribution of colony variants after "adaptation" to growth in heterologous serums. No further categorization beyond compact and diffuse was attempted in these experiments (*see* text).

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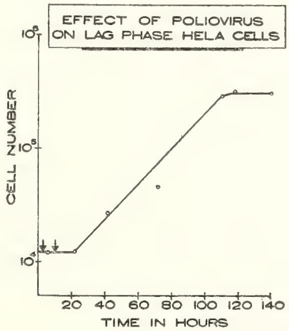
[5] Of the variant cell types in the 4 cell strains, 11 of 18 appear genetically stable under these primitive test conditions; the more refined analytical methods have been described by Vogt (*11*).

Table 7.—Colony morphology of cells propagated in heterologous serums

Colony cell type* of HeLa-S3	Colony cell type when propagated in Eagle's medium containing 20 percent serum:		
	Human	Calf	Horse
Compact-large Diffuse-large	Compact-large Diffuse-large	Compact-large Diffuse-large	Compact-large Diffuse-large

*Cell variants retained their distinctive colony characteristics regardless of the serum used in growth medium. Morphologic "modulation" occurred, *i.e.*, cells of the compact-large type were smaller when grown in horse serum than those propagated in human serum.

The heteroploid nature of mammalian cells in most strains makes it difficult to distinguish between genotypic and phenotypic changes in cell populations (*11*). Thus, a study of "clonal variation" and susceptibility of cells to virus infection is confounded by the difficult problem of differentiating between cell genotype and phenotype. However, by the use of suitable viruses and their infective nucleic acids, distinctions can be made between the genetic, biochemical, and physiologic competence of cells to become infected and replicate virus. The studies of Holland *et al.* (*30*) and Darnell and Sawyer (*31*) serve as suitable reference material. For the submoderate mammalian viruses, cellular susceptibility to infection can be an illusive quality that may or may not be causally related to infective attachment and penetration of viral nucleic acid (*31*). Moreover, Roizman (*32*) recently showed that the stage of cell mitosis can regulate the response of cells to infection. The influence of cell physiology on response of bacteria to infection by temperate bacteriophage (*33*) is another example of the manner in which phenotypic alterations in cells can influence virus-cell interaction. The data in text-figure 5 provide another example indicating how the physiologic status of mammalian cells can regulate their response to infection by submoderate



Text-figure 5.—The data illustrate how cell physiology (phenotypic changes) influences cellular response to virus infection. The curve represents typical results for the growth of a population of wild HeLa-Gey cells that normally are fully susceptible to cytopathic destruction by the test submoderate poliovirus. When submoderate virus was added to cells at a high multiplicity of infection during the first 8 hours of the lag phase of cell growth, cells were not destroyed during the following 8 hours. If virus was added during the *last* portion of the lag phase of growth (*arrow* on the *right*), complete cell destruction resulted.

poliovirus. The curve represents typical results for the growth of a population of wild HeLa-Gey cells. Since poliovirus, when used at high multiplicities of infection, requires only about 8 hours to destroy susceptible cells, it was possible to divide the lag phase of the naturally susceptible HeLa-Gey cells into two equal parts: In one, submoderate virus was added during the first 8 hours of the lag phase of growth; in parallel experiments, virus was added during the last portion of the lag phase. Under these conditions, cells exposed to virus during the first portion of the lag phase of growth failed to yield morphologic evidence of infection. In contrast, complete cytopathology resulted when virus was added during the last part of the lag phase. In all instances, however, virus eventually destroyed the test cell population. Similar findings have been noted with the parainfluenza viruses and with the virus of herpes simplex.

SUMMARY

A number of representative experimental findings have been presented and discussed: These illustrate the complex nature of the problems encountered in arriving at an analysis of "clonal variation and interaction of cells with viruses." Data from virus-cell culture studies were analyzed to illustrate the need for a more adequate characterization of cell populations so that experimental studies of cells in culture can be more informative.

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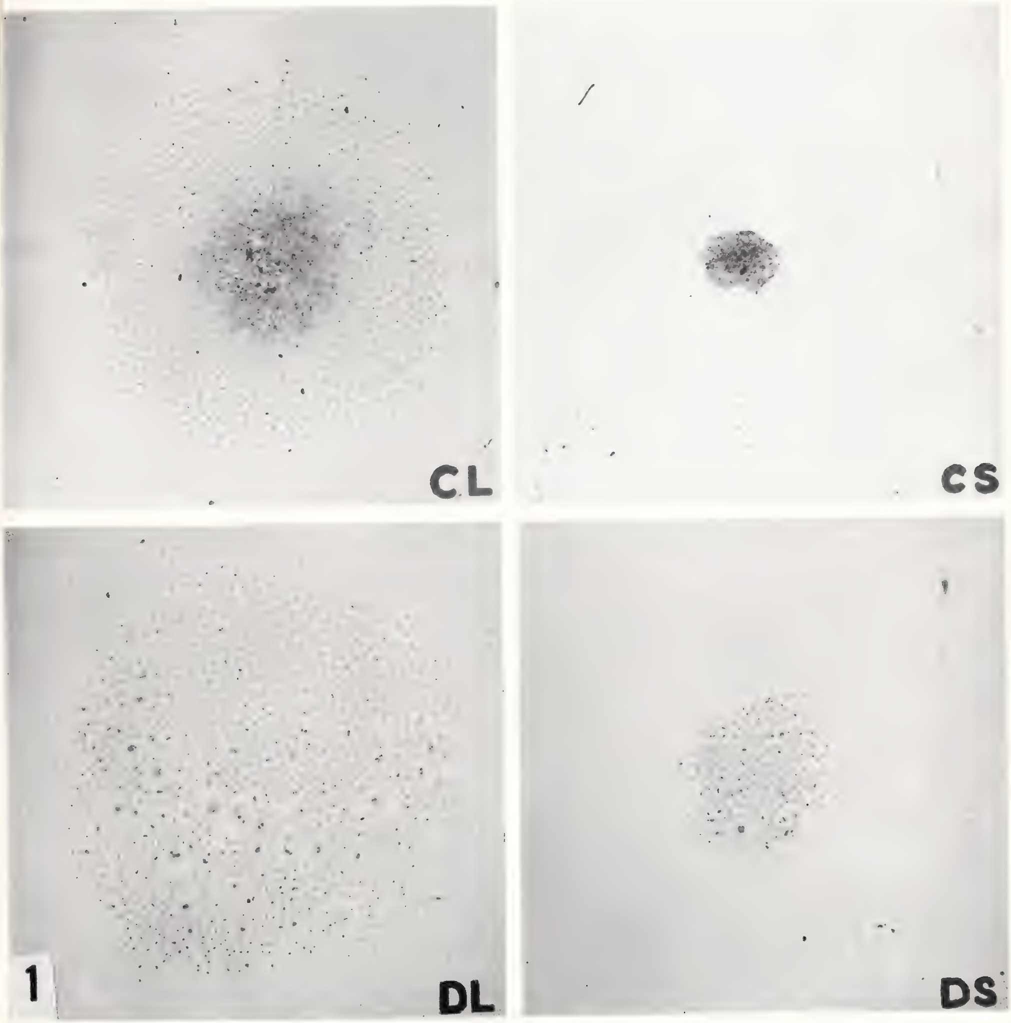
Plate 28

Figure 1.—Colonies developing from wild-cell populations of the 4 cell strains tested were: compact-large (CL), made up of round, epithelioid cells giving rise to multilayered opaque colonies; compact-small (CS), approximately ½ the size of the CL type; diffuse-large (DL), made up of fibroblastic cells; colonies contained many translucent giant cells; diffuse-small (DS), essentially half the size of the DL type. Colonies shown are from the HeLa-S3 strain. × 17


CL
CS
1
DL
DS

DISCUSSION

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Dr. Murphy has reviewed rather extensively an area of increasing interest in recent years; namely, variations in cell populations in relation to virus susceptibility. I would like to extend Dr. Murphy's data with some of our own on Chang's human liver cells and the psittacosis agent.

Figure 1 shows the two predominant clones Dr. Officer and I found in a pleuropneumonia-like organism (PPLO)-free line of Chang's human liver cells—one clone, compact and the other, diffuse.

Figures 2 and 3 show the results of infecting both clones in the same petri dish with a single dose of the moderately virulent 6BC strain of psittacosis virus. Most of the cells in the diffuse clone contain the specific virus inclusions in the cytoplasm, but few cells in the compact clone contain such inclusions.

Figures 4 and 5 show how the clonal type may also influence the inclusion-body morphology of the more virulent Borg strain of virus. Again both clones were infected with a single dose of virus in the same petri dish. It may be seen that this strain of virus forms diffusely spread elementary bodies and inclusion bodies in the diffuse clone. This is typical of the Borg strain in all the cell types we have studied. In the compact cell clone, however, the Borg strain forms compact inclusions. We believe the ability of the Borg strain to spread in a cell is related to its virulence (*1*, *2*). The cytologic data showing the influence of the clonal type on both strains of virus (figs. 2, 3, 4, and 5) are supported further by quantitative data, which we have published recently, on the percentage of cells of each clonal type that may be infected with a selected dose of virus and on differences in the virus growth curves obtained in the two different cell clones (*2*).

Our observations on a chronically infected culture of Chang's cells led to a study of the population genetics relating to this cell line. We found the following: The parent population was composed of approximately 80 percent compact clonal types and 20 percent diffuse types. The compact clones remained compact on repeated cloning and never gave rise to diffuse clones even when large populations of cells were permitted to grow from a number of single clones. In contrast to this, the diffuse clones, though remaining diffuse for the most part on repeated cloning, always gave rise to compact types when they were allowed to grow into large populations and cloned. Compact types began to overgrow the diffuse types but eventually stabilized at the same 80 percent compact and 20 percent diffuse type cells as in the parent population. On a few occasions we could observe the change from diffuse to compact in the early stages. Figure 6 shows the beginning formation of nests of compact cells in initially diffuse clones, which were genetically unstable in tissue-culture medium. Varying the conditions in tissue culture we have thus far failed to alter the results. However, by transplanting our parent cells into the brains of unconditioned mice, we obtained a pure culture of a stable diffuse clonal type from the resulting tumor after 10 days—a culture that did not give rise to compact clones and was as susceptible to psittacosis virus as the unstable diffuse type. The mouse-brain environment apparently selected a *stable* diffuse variant that could not be obtained in tissue culture. With the help of Dr. Pious in our laboratory, we have continued our studies by determining the stemline chromosome numbers of all our clonal types, which now number three diffuse and three compact types—each distinguishable genetically (*3*). While the time is too short to present the data in detail, our results thus far show that the characters of clonal morphology and resistance to psittacosis virus are associated, but the stemline chromosome number shows no constant association with either of the other two characters.

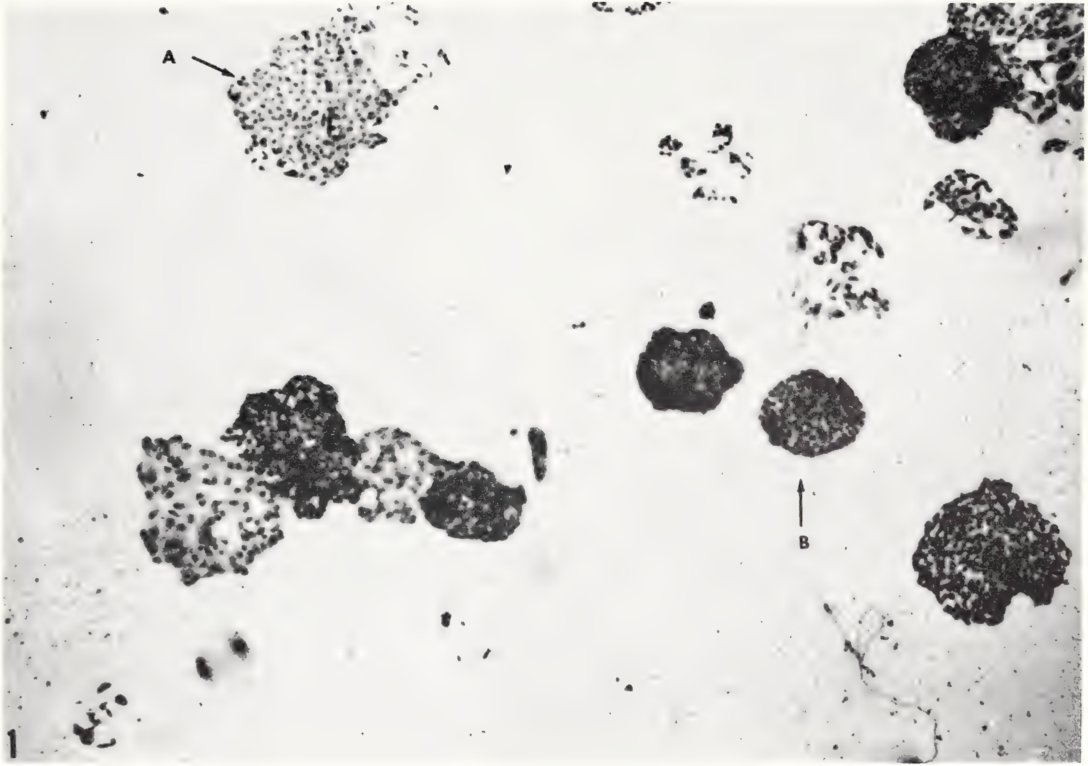
In summary, our studies show that a population of cells may be composed of a number of clonal types whose proportions may vary and/or be controlled by specific environmental conditions. The characters of clonal morphology and degree of resistance to psittacosis virus were associated, but neither of these was associated with the stemline chromosome number of the cell type.

References

(*1*) Officer, J. E., and Brown, A.: Growth of psittacosis virus in tissue culture. J. Infect. Dis. 107: 283–299, 1960.
(*2*) ———: Serial changes in virus and cells in cultures chronically infected with psittacosis virus. Virology 14: 88–99, 1961.
(*3*) Pious, D. A., Officer, J. E., and Brown, A.: Unpublished observations.

PLATE 29

FIGURE 1.—Clonal types existing in a parental population of Chang's human liver cells. *Note* two predominant types: A—CHL_S, diffuse, thinly spread; B—CHL_C, compact, sometimes in multiple cell layers (2). May-Grünwald-Giemsa stain × 75



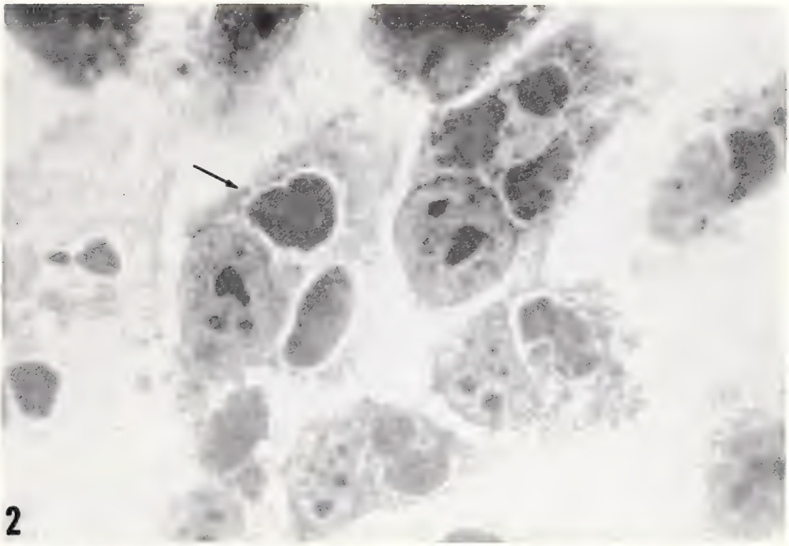
BROWN

PLATE 30

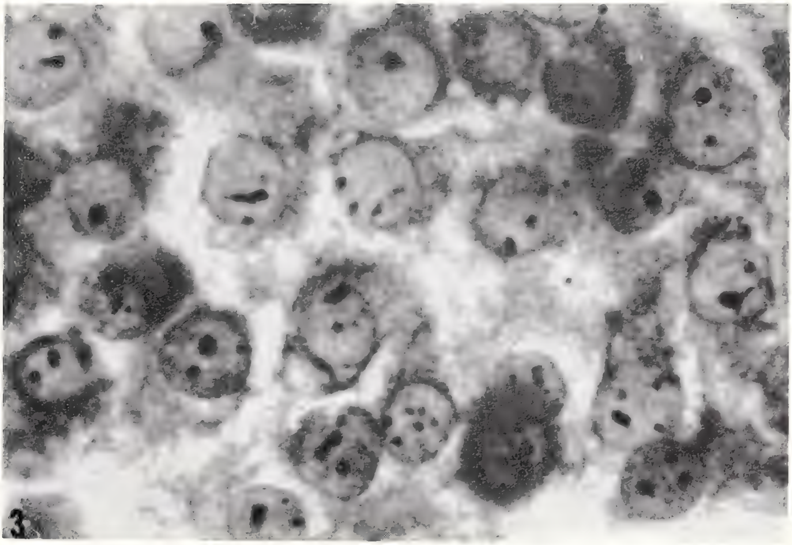
Both clones infected in same petri dish with single dose, 10^5 yolk sac LD50, of virus.

FIGURE 2.—Susceptibility of diffuse clone of Chang's human liver cells to psittacosis virus (6BC). *Note* virus inclusions (*arrow*) in contrast to few in compact clone [fig. 3 (*2*)]. May-Grünwald-Giemsa stain. × 2,800

FIGURE 3.—Susceptibility of compact clone of Chang's human liver cells to psittacosis virus (6BC). *Note* few virus inclusions (*2*). May-Grünwald-Giemsa stain. × 2,800



2



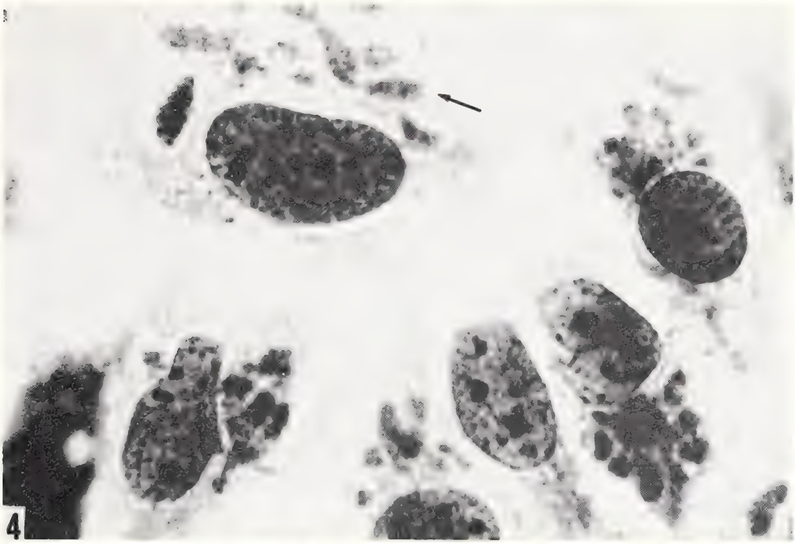
3

Plate 31

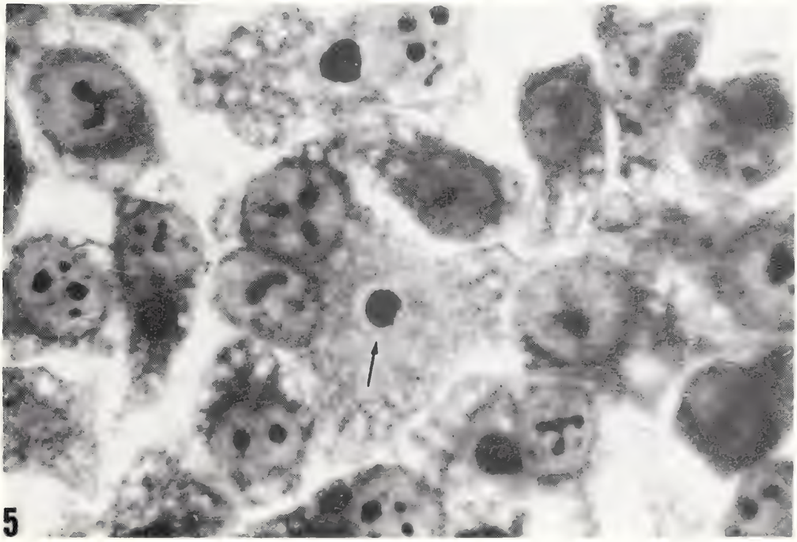
Both clones infected with 10^6 yolk sac LD50 of virus in same petri dish.

Figure 4.—Inclusion-body development of Borg strain of psittacosis virus in diffuse clone of Chang's human liver cells. *Note* difference in type of inclusion (*arrow*) in diffuse and compact clone [fig. 5 (*2*)]. May-Grünwald-Giemsa stain. × 2,800

Figure 5.—Inclusion-body development of Borg strain of psittacosis virus in compact clone of Chang's human liver cells. *Note* difference in type of inclusion (*arrow*) in compact and diffuse clone [fig. 4 (*2*)]. May-Grünwald-Giemsa stain. × 2,800



4

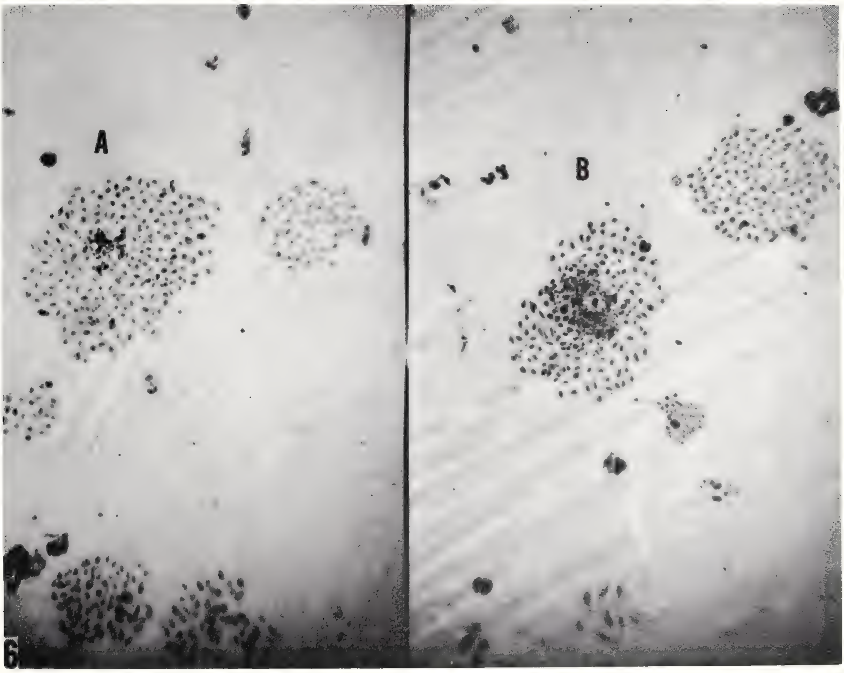


5

BROWN

Plate 32

Figure 6.—Nests of cells typical of compact clones arising in initially diffuse clones. A—beginning phase in a diffuse clone; B—later phase in another diffuse clone.


A
B
6

Cellular Factors Associated With Susceptibility and Resistance to Enteroviruses [1,2]

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IT HAS long been known that while poliovirus can infect many types of human and monkey cells *in vitro* with ultimate destructive effect, cells derived from naturally insusceptible nonprimate animals are resistant to infection by this virus. Possibly this apparent cellular resistance was correlated with failure to adsorb virus irreversibly as demonstrated with phage-resistant mutants of *Escherichia coli* B (*1*), or one or more of the subsequent stages in the infection cycle, such as penetration, replication and assembly of viral components, or liberation of virus into surrounding medium, might be the limiting factors. Studies with partially resistant primate cells showed a number of these possibilities may be operative in determining susceptibility and resistance. Kaplan (*2*) demonstrated that cultures of capuchin monkey kidney cells were only partially susceptible to poliovirus infection and that resistance of these cells might be explained by the fact significant amounts of virus were not adsorbed. The possibility of resistance mechanisms independent of adsorption have also been reported. Hsiung (*3*) found patas monkey kidney cells that are resistant to Coxsackie A-9 virus did adsorb virus, but the cells produced virus only after X irradiation. In addition, Vogt and Dulbecco (*4*) reported HeLa cells selected for resistance to poliovirus could adsorb virus and attributed the resistance to an apparent decreased probability that adsorbed virus could initiate infection in these cells. In a similar system, Darnell and Sawyer (*5*) showed that the partial resistance to poliovirus infection in one line of HeLa cells was attributable to inefficiency of the cells in effecting the release of viral ribonucleic acid (RNA) from its protein coat.

The studies described here concern the primary attachment of poliovirus to fully susceptible or resistant cells in cultures of primate and nonprimate origin, infection of naturally insusceptible cells by enterovirus RNA, and some properties of enteroviral "receptors" of HeLa cells.

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[2] Aided by grants from the National Foundation, the National Science Foundation, the American Cancer Society, Inc., and the National Cancer Institute, National Institutes of Health, Public Health Service.

EXPERIMENTS AND RESULTS

Poliovirus Adsorption to Primate and Nonprimate Cells

A variety of cell cultures derived from various animal species was examined for cytopathic response and for their ability to adsorb type 1 (Mahoney) poliovirus. The results are presented in table 1. All cell cultures derived from primates showed cytopathic degeneration and actively adsorbed poliovirus: however, with the exception of the embryonic rabbit kidney-1 (ERK-1) cell line, nonprimate cells neither adsorbed poliovirus to any extent nor responded cytopathologically to virus exposure. Culture fluids from unaffected cultures were passed serially through homologous cultures without any subsequent appearance of cytopathic effect or virus multiplication when assayed on HeLa cell monolayers. The ERK-1 cell was recently reported to be of human origin on the basis of antigenic (*6*, *7*) and chromosome (*8*) analyses.

TABLE 1.—Adsorption of poliovirus to cell cultures derived from several animal sources*

Cells†	Type culture	Source	Cytopathic effect observed	Virus adsorbed from 0.1 ml inoculum in 2 hours at 37° C
				Percent
HeLa	Continuous culture	Human	+	>90
Detroit-6	Continuous culture	Human	+	>90
Harris	Continuous culture	Human	+	>90
Esophageal epithelium	Continuous culture	Human	+	>90
Amnion	Primary culture	Human	+	>90
Kidney	Primary culture	Human	+	>90
Kidney	Primary culture	Monkey	+	>90
Kidney (ERK-1)	Continuous culture	Rabbit	+	>90
Kidney (ERK-2)	Continuous culture	Rabbit	0	<10‡
Kidney	Primary culture	Rabbit	0	<10
Kidney	Primary culture	Dog	0	<10
Kidney	Primary culture	Cat	0	<10
Kidney	Primary culture	Swine	0	<10
Kidney	Primary culture	Calf	0	<10
Kidney	Primary culture	Guinea pig	0	<10
Embryonic, mixed	Primary culture	Mouse	0	<10
Embryonic, mixed	Primary culture	Chick	0	<10
Papilloma (cottontail rabbit papilloma)	Continuous culture	Rabbit	0	<10
Skin (domestic rabbit fibroblast)	Continuous culture	Rabbit	0	<10
Skin (cottontail rabbit epithelium)	Continuous culture	Rabbit	0	<10
Strain L	Continuous culture	Mouse	0	<10

*Reproduced from J. Exper. Med. 109: 479, May 1959.

†Approximately 2 × 10^6 cells per monolayer.

‡No adsorption within limits of method.

Fate of Virus Adsorbed by Susceptible and Insusceptible Cells

To determine whether virus was adsorbed to and multiplied within only a small proportion of susceptible cells in the nonprimate cultures, monolayers were exposed to high multiplicities of poliovirus. Time was allowed for viral adsorption and then the monolayers were rinsed thoroughly and trypsinized. The dispersed cells were enumerated, diluted, and plated on HeLa monolayers to determine the number of infected cells. To ascertain cell-associated or unproductively adsorbed virus within or on cells, we disrupted samples of the virus-treated cell suspensions by freezing and thawing before further incubation. Results in table 2 show that HeLa and monkey kidney cells, which actively adsorb virus, became infected in high proportion, yet they yielded very little infectious virus when disrupted soon after virus exposure. This ability of susceptible cells to eclipse poliovirus was demonstrated previously (*9–11*). In contrast, cells that did not adsorb significant amounts of virus such as calf and rabbit cells yielded only the same small amount of virus whether disrupted or left intact. Some virus was thus received by insusceptible cells but did not enter an eclipse phase as did virus received by susceptible cells. Further studies on unproductively (*i.e.*, virus that is not eclipsed by cells) adsorbed, noneclipsed poliovirus associated with susceptible or insusceptible cells showed that it represents only about 1 percent of the input virus, and that 5 to 16 percent of this virus is antiserum-resistant (*12*). Thus it is apparent that a small amount of poliovirus becomes cell-associated at the time of virus exposure, as previously reported for influenza virus by Ackermann and coworkers (*13*) and for Newcastle disease virus by Rubin *et al.* (*14*). Because only part of the unproductively adsorbed poliovirus could be neutralized by antiserum after cell disruption, it is indicated that virus is in some way associated with the cell surface and thus is inaccessible to antibody.

TABLE 2.—Proportion of cells giving rise to plaques after exposure to poliovirus at high multiplicity*

Cell culture	Source	Treatment	Cells producing plaques (percent)
HeLa	Human	None	70–95
		Disrupted†	0.1–1.0
Kidney	Monkey	None	50–90
Kidney	Monkey	Disrupted	<0.1
Kidney	Calf	None	<0.1
Kidney	Calf	Disrupted	<0.1
Kidney	Rabbit	None	<0.1
Kidney	Rabbit	Disrupted	<0.1
Papilloma (cottontail rabbit papilloma)	Rabbit	None	<0.1
Papilloma (cottontail rabbit papilloma)	Rabbit	Disrupted	<0.1
Skin (domestic rabbit fibroblasts)	Rabbit	None	<0.1
Skin (domestic rabbit fibroblasts)	Rabbit	Disrupted	<0.1

*Reproduced from J. Exper. Med. 109: 480, May 1959.

†After cells were enumerated, we disrupted a sample by freezing and thawing during the eclipse phase, *i.e.*, 2.5 hours after exposure.

Effect of Physiologic State of HeLa Cells on Their Ability To Become Infected by Poliovirus

Once poliovirus has become adsorbed to HeLa cells, the rate of penetration of virus (*i.e.*, the rate at which adsorbed virus becomes antiserum-resistant) is temperature-sensitive (*15*, *16*). Most adsorbed virus remains antiserum-sensitive if the monolayers are maintained at 1° C after adsorption instead of 37° C. Thus at 1° C adsorbed virus is held at the cell surface where it is susceptible to antiserum, but at 37° C it penetrates beyond the sphere of antiserum influence (*16*).

To determine further the role of cellular physiologic state on the initiation of infection other procedures were also investigated. HeLa cells were heat-killed by exposure to 56° C for 2 minutes, starved by storage in balanced salt solution (BSS) at room temperature for 5 days, poisoned by exposure to sodium azide, or left untreated as controls. In comparison to control cultures, damage of the treated cells resulted in reducing the plating efficiency to less than 0.1 percent, yet the same proportion of virus (more than 95%) was inactivated—an indication that unimpaired cellular physiology is not essential to virus eclipse.

To determine whether cells must be structurally intact to effect this apparent virus eclipse, we disrupted HeLa cells by 10 cycles of freezing and thawing, and debris prepared from these cells at a concentration of 5×10^6 cells per ml was mixed with virus. It was found that the debris could actively bind virus (table 3). Furthermore, this antiviral effect of cell debris was correlated with poliovirus susceptibility. Debris prepared from all susceptible cells inactivated virus, whereas debris prepared from resistant, nonadsorbing cells failed to inactivate significant amounts of virus under the same conditions [table 4, (*16*)].

Infection of Naturally Insusceptible Cells With Enteroviral RNA

It was of interest to learn whether the specific capacity of primate cells to receive, replicate, and release poliovirus was conditioned entirely by the presence of the virus-binding or receptor mechanism. Alexander and coworkers (*17*) had shown that HeLa cells could be infected by poliovirus RNA, and with this information we attempted to circumvent the primary interaction of receptor and viral protein by exposing insusceptible cells to poliovirus RNA prepared by the Gierer-Schramm method which Alexander *et al.* (*17*) had used. A summary of the results (table 5) indicates that it

TABLE 3.—Inactivation of poliovirus by debris from disrupted HeLa cells* †

Reaction mixture	Plaques/monolayer
Type 1 poliovirus+BSS (control)	117, 124, 109, 116, 122
Type 1 poliovirus+HeLa debris	3, 1, 2, 4, 2
Type 2 poliovirus+BSS (control)	87, 78, 91, 83, 87
Type 2 poliovirus+HeLa debris	0, 1, 3, 1, 2

*Reproduced from J. Exper. Med. 109: 494, May 1959.

†Virus was incubated at 37° C for 2 hours in 1.0 ml BSS (control) or in 1.0 ml BSS containing debris from 5×10^6 disrupted HeLa cells. After 2 hours, mixtures were diluted 1:100 and 0.1 ml portions were plaque-assayed for remaining virus.

TABLE 4.—Inactivation of type 1 poliovirus by debris from susceptible and insusceptible mammalian cells*

Cell and species source of debris	Poliovirus inactivated by cell debris† (percent)
HeLa (human)	98
Harris (human)	88
HeLa grown in calf serum	92
Esophageal epithelium (human)	>99
Detroit-6 (human)	92
Primary human amnion	96
Primary monkey kidney	>99
ERK-1 (transformed rabbit cell)	98
ERK-2 (untransformed rabbit cell)	0
Domestic rabbit fibroblasts	0
Cottontail rabbit epithelium	0
Cottontail rabbit papilloma	0
Strain L mouse fibroblasts	0
Primary calf kidney	0
Primary rabbit kidney	0
Primary guinea-pig kidney	0
HeLa + domestic rabbit fibroblast	95
HeLa + primary calf kidney	97

*Reproduced from J. Exper. Med. 109: 494, May 1959.

†Percent poliovirus losing infectivity after 2 hours' incubation at 37° C with debris from 5×10^6 cells of the type indicated.

TABLE 5.—Production of type 1 poliovirus by insusceptible cells inoculated with viral RNA*

Test system†	Total plaque-forming units per culture vessel 18 hours after inoculation of poliovirus RNA‡	
	Experiment A	Experiment B
Susceptible cell control:		
HeLa	5×10^4	4×10^3
Established lines of nonprimate cells:		
Domestic rabbit fibroblast (Minn. 56–8–6)	7×10^4	9×10^2
Cottontail rabbit epithelium (Minn. 57–8–19)	2×10^3	4×10^2
Cottontail rabbit epithelium (Minn. 56–8–8)	0	3×10^2
ERK-2 (Westwood's normal rabbit kidney cell)	2×10^5	4×10^3
Strain L mouse fibroblasts	5×10^2	8×10^4
Primary monolayer cultures of nonprimate cells:		
Rabbit skin	4×10^3	9×10^4
Rabbit kidney	6×10^4	5×10^2
Guinea-pig kidney	0	1×10^4
Swine lung	2×10^3	5×10^4
Embryonic mouse carcass	8×10^3	—
Maitland-type cultures		
Rabbit liver	3×10^3	—
Guinea-pig liver	4×10^5	1×10^4
Hamster liver	9×10^2	—
Mouse liver	2×10^2	5×10^3
Chick liver	0	7×10^3
Minced chick embryo	1×10^3	5×10^2

*Reproduced from J. Exper. Med. 110: 72, July 1959.

†Monolayer cultures of about 2×10^6 cells were inoculated with 0.1 ml of RNA; 0.5 ml was added to washed tissue fragments in Maitland-type cultures.

‡No poliovirus was produced when RNA was treated with ribonuclease before inoculation, or when cultures were harvested 3 hours after inoculation.

was possible to cause poliovirus replication and release in naturally insusceptible cells in this way. Also, viral multiplication takes place after inoculation of cell cultures or when naturally resistant animals are injected intracerebrally with RNA prepared from poliovirus [table 6 (*18*)]. Mountain and Alexander (*19*) have also reported infection of chick embryos with RNA from type 1 poliovirus, and recently Gordon and Smith (*20*) reported infection of insusceptible plants with tobacco-mosaic virus RNA. The ability of poliovirus RNA to infect cells from every warm-blooded animal species tested suggests that many if not all mammalian cells may possess the metabolic capacity for poliovirus synthesis. Therefore, natural susceptibility of primate cells to infection by intact poliovirus may be determined by the primary interaction of this receptor-like substance and virus. This is not to say, however, that susceptibility *in vivo* is not affected by hormonal, immunologic, and physiologic factors which may

TABLE 6.—Production of type 1 poliovirus by animals inoculated with viral RNA*

	Total plaque-forming unit/ culture vessel 18 hours after inoculation of poliovirus RNA‡	
Test system†	Experiment A	Experiment B
Living animals inoculated intracerebrally:		
Rabbit	4×10^3	—
Guinea pig	3×10^4	8×10^2
Hamster	6×10^3	—
Chick	2×10^3	0

*Reproduced by J. Exper. Med. 110: 72, July 1959.

† RNA, 0.03 ml, was injected intracerebrally into mice, and 0.05 ml into the larger animals, and after 18 hours brain tissue was assayed for poliovirus on HeLa monolayers.

‡ No poliovirus was produced when RNA was treated with ribonuclease before inoculation, or when brains were harvested 3 hours after inoculation.

TABLE 7.—Inactivation of Coxsackie A-9 virus by debris from disrupted cells*

		Virus inactivation†		
Source of cell debris	Type of culture	Coxsackie A-9	Poliovirus type 1	Coxsackie B-1, -3, -5
Human amnion	Primary	+‡	+	+
Monkey kidney	Primary	+	+	+
HeLa	Continuous	0§	+	+
KB	Continuous	0	+	+
Human amnion	Continuous	0	+	+
Human chorion	Continuous	0	+	+
Human kidney	Continuous	0	+	+
Monkey kidney + human kidney	Primary + continuous	+	+	+
Rabbit papilloma	Continuous	0	0	0
Heated amnion||	Primary	0	0	0

*Reproduced from J. Exper. Med. 112: 587, October 1960.

† Viruses were incubated at 37° C for 2 hours in BSS (control) or in 1 ml BSS containing debris from 5×10^6 disrupted cells. After 2 hours mixtures were diluted 1:100 and 0.1 ml samples were plaque-assayed for remaining virus.

‡ +, 80 Percent or more loss of viral infectivity.

§ 0, No measurable loss of viral infectivity.

|| Debris from amnion cells heated to 56° C for 30 minutes.

also influence entrance of virus into cells and subsequent intracellular viral synthesis.

Specificity of Inactivation of Enteroviruses by Cell Debris

It is well known that whereas primary human amnion and monkey kidney cells are extremely susceptible to Coxsackie A-9 virus, numerous strains of HeLa cells and other human cells in continuous culture are either relatively or completely resistant to this virus. Using 8 different stable cell lines of human origin, we found that none were destroyed after exposure to Coxsackie A-9 virus as were primary amnion or monkey kidney cells. All these cells, however, were destroyed by Coxsackie B-1, -3, and -5 viruses. Results shown in table 7 indicate that addition of cell debris prepared from the stable cells to these strains of Coxsackie virus and to type 1 poliovirus results in adsorption of all but Coxsackie A-9 virus (*21*). Again, there was good correlation between virus adsorption by cell debris and susceptibility of intact cells.

Reversibility of Enterovirus Inactivation by Cell Debris

It was of interest to know whether the inactivation of enteroviruses by debris from susceptible cells was reversible or whether some physical or chemical alteration of adsorbed virus with permanent impairment of infectivity occurred. It was found that debris-inactivated virus could not be recovered by dilution, freezing, and thawing, or sonic treatment; however, with Coxsackie A-9 virus, infectious virus could be completely dissociated from primary human amnion cell debris at *p*H 4.5 (*21*). Mandel (*15*) had reported this method as effecting the dissociation of poliovirus from homologous antibody. Treatment of cell debris-inactivated poliovirus at this *p*H would not release virus from debris, but *p*H 2.5 after 1 hour at 37° C was effective (*21*). Recently we found that treatment of poliovirus-cell debris mixtures with a variety of substances that inactivate receptor substance will result in dissociation of poliovirus. Some of those substances are 8 M urea, sodium dodecyl sulfate, and sodium deoxycholate (*22*). Thus, the primary interaction of cell debris and the viruses tested does not result in true eclipse of infectivity.

Properties of the Poliovirus Inactivating Component of HeLa Cell Debris

Original studies on the nature of the viral-binding factor in HeLa cell debris indicated that most of the activity was sedimentable within 20 minutes at 10,000 × *g* but not at 1,000 × *g* for the same time (*16*). Furthermore, activity was not lost by repeated washing of the sedimentable fraction. Preliminary attempts to characterize receptor substance have been made, and the effects of a number of chemical and physical treatments on the antiviral activity of HeLa cell debris are presented in table 8. It is apparent that activity is lost by heating, treatment with lipide solvents, protein denaturants, and trypsin and by prolonged sonic disruption but not by exposure to lipase, periodate, or *Vibrio comma* receptor-destroying enzyme (*16*). These results suggested that the receptor substance was possibly an insoluble lipoprotein associated with the cell membranes. The reported loss of activity by sonication was the result of thermal inactivation, because we recently found that if the temperature is maintained near 0° C sonic disruption does not destroy antiviral activity. However, all other attempts so far to solubilize receptor from cell debris, for example, treatment with 0.1 N HCl or 0.1 N NaOH, sodium deoxycholate and sodium dodecyl sulfate have resulted in loss of activity (*22*). Our data now indicate that protein is an essential component of the enteroviral receptor; however, it has not been possible to determine whether lipide definitely plays a role in virus binding. Activity is not lost after treatment with Tween 20 and Tween 80 which may mean that loosely bound lipides are not essential for receptor function (*22*). It is of interest that evidence presented by Few and coworkers (*23*) *et al.* indicates that lipides of some bacterial lipoprotein cell membranes apparently are not exposed at the surface but are sheathed by proteins.

TABLE 8.—Effect of chemical and physical treatment on antiviral activity of HeLa cell debris*

Treatment	Poliovirus inactivated by treated debris at 37° C in 2 hours
	percent
None	>99
Heated 56° C 15 minutes	0
Incubation at 37° C for 24 hours	0
Extracted with ether	0
Extracted with chloroform	0
Periodate	>99
Trypsin	0
Lipase	>99
Receptor-destroying enzyme	>99
Sonic disruption for 15 minutes	0

*Reproduced from J. Exper. Med. 109: 497, May 1959.

In attempts to purify the active component of debris, subcellular fractions of HeLa and strain L cells were prepared according to a modification of methods described by Schneider (*24*), with 0.25 M sucrose as the suspending medium. Although weak activity is associated with the nuclear, mitochondrial, and postmicrosomal fractions of HeLa cells, the microsomal fraction is most active in the inactivation of poliovirus and Coxsackie B-1 virus (*22*). No antiviral activity has been demonstrated in any fractions prepared by the same method from strain L cells which are insusceptible to infection.

We have also tested the microsomal fraction prepared from HeLa cells for capacity to adsorb vaccinia and Newcastle disease virus. No binding of these viruses with the microsomal fraction or crude cell debris was detected, though both viruses attach to intact HeLa cell monolayers.

Occurrence of Poliovirus Receptors *In Vivo*

We tested a variety of tissue homogenates prepared from human fetal organs, as well as normal and malignant adult human tissues, for the presence of poliovirus receptor. According to Kaplan (*2*), when poliovirus was inoculated *in vivo* into the kidney of the rhesus monkey, no viral multiplication could be detected. This was surprising because primary cell cultures prepared from rhesus kidney are fully susceptible to infection by poliovirus. By using homogenates prepared from organs of 5 different human fetuses, we found that the only preparations actively adsorbing poliovirus were from brain, spinal cord, and small intestine (table 9). Furthermore, only microsomal fractions prepared from these organs showed activity. Similar results were obtained when normal adult human tissues were used; however, two different malignant tissues, a breast carcinoma and a fibrosarcoma of the brain, adsorbed virus. In 1953 Francis and Chu (*25*) found, while studying the mode of injury produced by the Lansing strain of poliovirus, that only homogenates prepared from the gray matter of the central nervous systems of man and the rhesus monkey interacted with poliovirus. Gastrointestinal-tract tissues were not studied. Sabin (*26*) also found that gray matter from cynomolgus monkeys, chimpanzees, and man combined with type 1 poliovirus, while that of the dog did not. Recently, Kunin and coworkers (*27*) reported that homogenates prepared from a variety of rhesus monkey tissues, as

TABLES 9.—Inactivation of type 1 poliovirus by homogenates and microsomal fractions of some human fetal tissues* †

Tissue	Type preparation	Number tested	Number inactivating virus	Percent virus inactivated
Brain	Homogenate	5	3	>90
Spinal cord	Homogenate	3	3	>90
Small intestine	Homogenate	5	5	>90
Large intestine	Homogenate	3	0	<10
Stomach	Homogenate	3	0	<10
Spleen	Homogenate	4	0	<10
Lung	Homogenate	5	0	<10
Liver	Homogenate	5	0	<10
Muscle	Homogenate	5	0	<10
Skin	Homogenate	5	0	<10
Pancreas	Homogenate	3	0	<10
Thymus	Homogenate	2	0	<10
Brain	Microsomal fraction	3	3	>90
Spinal cord	Microsomal fraction	3	3	>90
Small intestine	Microsomal fraction	5	5	>90
Lung	Microsomal fraction	3	0	<10
Muscle	Microsomal fraction	3	0	<10

*Virus was incubated at 37° C for 2 hours in 1.0 ml BSS (control) or in 1.0 ml BSS containing tissue debris or microsomal fractions. After 2 hours, mixtures were diluted 1:100 and 0.1 ml aliquots were plaque-assayed for remaining virus.

†Results presented in this table were obtained independently at the University of Minnesota and the University of Washington.

well as normal and malignant human tissues, would actively adsorb poliovirus. However, with mouse tissue minces and Coxsackie B-1 virus, only brain tissue contained demonstrable receptor activity for this virus (*28*). At the present time, it is not possible to state whether the apparent tropism of these enteroviruses *in vivo* is determined by the qualitative or quantitative distribution of specific receptors in various tissues. Lack of agreement regarding their presence in insusceptible tissues, such as lung, heart, and testis, may result from the use of different techniques. Furthermore, some tissues may lose activity because of sensitivity to thermal inactivation or proteolytic enzymes, as indicated earlier. The results are in agreement, however, that poliovirus receptor activity is present in organs known to be important in the pathogenesis of poliomyelitis.

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DISCUSSION

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Yesterday a friend told me that as a discussant only the older men in virology could acceptably philosophize—the younger ones were expected to present their own data. I have no slides, no mass of pertinent or impertinent data of my own to interject into these few minutes. With this perhaps disturbing thought, I would like to discuss certain aspects of Dr. McLaren's very nicely presented systematic study.

In regard to the inactivation of poliovirus by certain tissues and not by others and the possible relationship of this to the pathogenesis of poliomyelitis, I would be inclined toward a liberal interpretation of Dr. McLaren's findings (*1*, *2*), those of Francis and Chu (*3*), and those of Sabin (*4*). All of them found that the *in vivo* susceptibility of cells or tissues was related to the *in vitro* ability of these to adsorb or inactivate virus. Kunin and Jordan (*5*) in preliminary studies using adult human tissues found adsorption of poliovirus limited to certain cells of the central nervous, lymphatic, and enteric tissues.

However, in contrast to Dr. McLaren's results, these same authors (*5*) found that a variety of tissues including amnion from a human fetus of 3-month gestation actively adsorbed virus. Full-term amnion failed to adsorb. This suggests that the fetal tissues examined by Dr. McLaren (*2*) might have been more mature and their cells were more highly differentiated than those studied by Kunin and Jordan (*5*). Both groups of investigators noted that malignant tissues adsorbed poliovirus, reminding us of the frequent comparisons made between neoplastic and embryonal cells.

Kunin and Jordan (*5*) also reported that a wide variety of tissues from rhesus monkeys adsorbed poliovirus and compared this finding with Wenner's observation (*6*) of widespread distribution of virus in tissues of cynomolgus monkeys following intramuscular or alimentary infection. The apparent lack of agreement regarding the adsorption of poliovirus by such tissues as lung, heart, liver, etc. should be considered not only in terms of the possible experimental variables mentioned by Dr. McLaren but also in terms of how little we know of the *in vivo* interaction of this virus with a given tissue of a given age in a given species. Also, the possibility remains that in some tissues *in vivo* cell-virus interaction is limited at some stage subsequent to adsorption.

I can understand why Dr. McLaren looked for a particular chemical entity in the active cell debris. Perhaps there were thoughts of similar compounds being used for chemotherapy, or perhaps this hypothetical compound, acting in the early stages of viral replication, would be susceptible to induced alterations. However, the apparent electrostatic nature of poliovirus adsorption (*7*), and the heretofore unsuccessful attempts to solubilize the inactivating substance (*8*), would lead one at least to consider the possibility that this is a physical or structural property of the microsomal fraction rather than a specific chemical entity.

What does this viral inactivation mean in terms of the initiation of viral replication? By analogy with the bacteriophages and because some animal viruses have been shown to disintegrate shortly after penetration (*9–11*), we suspect that the viral nucleic acid is freed from its protein during what was referred to as a "true eclipse." Operationally, a virus is in the eclipse phase when its infectivity is no longer demonstrable by the technique used. As Dr. McLaren has shown, such a definition is rather precarious, since by treating cell debris with, for example, deoxycholate, previously masked virus is made detectable. However, one wonders if the microsomal fraction of susceptible cells, having carried out what seems to be at least the first step in virus-cell interaction, might if reinforced, *e.g.*, with an energy generating system, then be able to carry the sequence further and yield free viral nucleic acid.

As noted by Dr. McLaren, some cells are known to be resistant to infection by viruses for reasons other than the inability to adsorb virus. One might expect, too, that variations could exist in the virus as well as the cell to yield a nonreacting system. Indeed, Sabin has reported (*4*) that differences exist between highly neurotropic and highly attenuated strains of poliovirus in their ability to combine *in vitro* with the gray matter of cynomolgus monkeys, chimpanzees, and man.

Lastly, as Dr. Hull (*12*) demonstrated earlier, susceptibility to viruses may be used to characterize cell lines and virus adsorption, in particular, might allow further characterization of those cells resistant to the various agents.

Further, many of us have been intrigued by the cellular reaction, rather confusingly termed "transformation" or "alteration," *i.e.*, when cultures yield continuous cell lines and in some instances show altered cellular morphology. Dr. McLaren's observations (*13*) on Coxsackie A-9 virus, and the development of cellular resistance through loss of ability to adsorb, offer an exacting method for detecting alterations in cell surface during the transformation of primate cells. Pulvertaft (*14*) using ECHO-6 and ECHO-9 viruses in primary and transformed thyroid cells and Beeukes (*15*) using ECHO-9 in primary and transformed human amnion cells noted similar alterations in susceptibility to virus. A reversal of this situation was seen when Coxsackie A-13 virus infected continuous lines of thyroid cells but not primary thyroid cultures (*14*). Similarly, very young cultures of kidney and amnion cells were found to be relatively resistant to poliovirus (*16–18*). In some of these instances, cellular resistance would seem to be related to viral adsorption; in others some stage of viral replication other than adsorption may be defective. We hope that in the near future the nature of the cell-virus interaction in each of these examples will be more clearly defined, as Dr. McLaren and his collaborators accomplished for the systems they investigated.

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CONCLUDING REMARKS [1]

HARVEY I. SCUDDER, ***Ph.D., Executive Secretary, Viruses and Cancer Board, National Cancer Institute,***[2] ***Bethesda, Maryland***

THE PRIVILEGE of presenting the closing statements of this meeting was extended to Dr. Stanhope Bayne-Jones, Chairman of the Viruses and Cancer Board, which serves the National Advisory Cancer Council. With regrets that he cannot be with you today, Dr. Bayne-Jones has asked that as Executive Secretary of the Board I substitute for him. I am indeed honored, but in consideration of these two very full and most excellent days of scientific papers and discussion, find it would be beyond my capability to assess critically in any degree the values and accomplishments of this meeting. Rather, I shall present some generalities I hope may prove valid and also give you, as background for the Symposium, an account of the rapid development of our Viruses and Cancer Program in which the art and science of cell culture is so vitally important, now and for the future.

First of all, in reverent remembrance of our fine friend and colleague, let me say that unquestionably Dr. Syverton would have been well pleased with this Symposium, to which he devoted a great measure of his time and energy. Through the many references to his work and through the papers by scientists associated with him we have all felt his presence here in these past two days. The plans he initiated and developed for the Symposium were carried to a very successful completion. Through the diligent efforts of those concerned with the program arrangements, the excellence of the papers and discussions, and the appreciation of this audience, all have shared in a most successful Symposium.

From its early status as a technique, cell culture has grown in complexity as its problems and its utility have been made the subject of research by an ever widening diversification of specialists. The papers presented here have covered the astounding range from classic morphology to the macromolecular study of surface antigens and enzyme systems, from hamster cheek pouch studies of transplanted cells to the production of polio ribonucleic acid. Cell culture has brought together and made necessary an integration of many disciplines, all of which have, in the living cell, business in common. The research now involving the culture of living cells has created a scientific watering place, to which come the cytologist, the geneticist, the biochemist, the virologist, the pathologist, the biophysicist, the immunologist, and many others.

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[1] Presented at the Syverton Memorial Symposium on Analytic Cell Culture, Detroit, Mich., June 6 and 7, 1961.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

The healthy influx of these many disciplines has been very evident in the papers presented in this meeting. Through such contributions, the witchcraft of cell culture is rapidly being replaced by the careful delineation of factors in cellular biology. This is now the beginning of an ever increasing quantitation in all aspects of cell study, with more sophisticated experimentation, and more closely controlled analyses of the dynamic events in the living cell than were considered possible but a few short years ago. Yet far more seems probable with the aid of the remarkable instrumentation and the microchemical and biological methodology now available.

Any pattern of growth, whether of a living thing or a discipline, suffers differential rates which call occasionally for drastic readjustment to maintain a favorable balance. This is true now for cell culture, realized most urgently in the need for revision in the quality of the standards for cell lines themselves and for the conditions of their maintenance. To assist all investigators by making available cell lines that have been characterized and certified, a Cell Culture Collection Committee was set up in early 1960 under the chairmanship of Dr. Jerome T. Syverton. Through his leadership and the cooperation of many of you, a program is now well under way which should soon result in the production of certified seed stock for the principal cell line now in use.

Cell lines free of microbial contaminants, and to the extent possible, of latent viruses, are being subjected to such characterization tests as species of origin, morphology and growth rates in standard media, chromosome number and stability, susceptibility and response to certain viruses, antigenic constitution, and viability under conditions of controlled freezing, storage, and thawing essential to the long-term preservation of such certified stock.

The first papers of the Symposium described in detail the plans and work of the Cell Culture Collection Committee—now under the chairmanship of Dr. William F. Scherer—of the cooperating laboratories, and the American Type Culture Collection which would hold and distribute these important certified seed stocks.

The Viruses and Cancer Board of the National Cancer Institute regards the development of certified cell lines as a most important advance, notably essential to the virologist in his search for the etiologic agents of human cancer, but also much needed by many investigators in other lines of research.

Development of the Cell Culture Collection Committee and of its parent, the Viruses and Cancer Board, may be of interest and hopefully of benefit to all of you engaged in cell culture and related research.

The new program of aid and encouragement in cancer and virus research now being carried forward by the National Cancer Institute had its inception in early 1958, with Dr. Wendell Stanley's testimony before Congress, urging that emphasis be given to research in virology directed toward the cancer problem. Beginning with the fiscal year 1959, funds were therefore made available in the budget of the National Cancer

Institute, and, through the Virology and Rickettsiology Study Section and the National Advisory Cancer Council, a renewed emphasis was brought to bear on the old and time-worn thesis of the viral etiology of cancer.

In February, 1959, the Viruses and Cancer Panel, now the Board, was set up under the chairmanship of Dr. Charles A. Evans and charged with the responsibility of advising the Council and the staff. Dr. Syverton, as one of its most active members, was responsible for beginning one of its first activities, that of seeking the means for developing certified cell lines.

Our new group of advisors defined many areas in addition to cell culture that would require support and encouragement if the research in human cancer were to advance through virology. Among the more pressing needs is that of the availability of reagents for the identification of viruses. To make possible such standard reagents, the National Cancer Institute has undertaken the support of Dr. Herbert A. Wenner's laboratory, in Kansas City, which for eight years has been the mainstay of the National Foundation's research on poliomyelitis. Through Dr. Wenner we hope to make available reference reagents for all of the enteroviruses of man. We are, at the same time, encouraging a large scale production of these on a commercial basis. In cooperation with other Institutes at the National Institutes of Health, we hope to secure a rather complete battery of identification reagents, both antigens and antiserums, for the human viruses. These reagents are required for the identification of the many viruses one may isolate in the quest for and in the elimination of agents associated with human cancer.

To study viruses in laboratory animals, the virologist also must have, in addition to characterized cell lines and identification reagents, an animal system which has been defined with regard to its viruses, and as far as possible, is virus-free. Accordingly, on advice of the Viruses and Cancer Board, a program for the viral definition of the laboratory mouse has been set up. This program has as its ultimate goal a defined subhuman primate for cancer-virus studies.

As a further resource required for pursuit of this difficult problem, human tissue—both normal and malignant, adult and embryonic—must be made available for these investigations. We have, therefore, initiated a program in this area. To round out this rather broad venture, we are also including a program of information services through which we hope to improve the use of literature and the general communication of the highly diverse disciplines that must be carefully brought together to achieve success in this new area.

The concern of the National Cancer Institute with the new Viruses and Cancer Program is primarily with making available to our grantees and other investigators such resources as will help insure the success of this most recent renewed interest in the thesis that viruses cause cancer. At the present time, the National Cancer Institute alone, in virology and related fields, is supporting 153 projects in the amount of 5.6 million dollars for this fiscal year.

We face the certain need for an increased manpower in this complex group of disciplines. It is particularly fitting that Dr. Syverton initiated a plan for centers of advanced training which has been approved in principle by the National Advisory Cancer Council and awaits our efforts to implement it. He envisioned a teaching environment in which all the research disciplines which impinge on cell biology can jointly contribute to the training of young scientists and to the additional training of senior people.

As a closing statement, made on behalf of the Viruses and Cancer Program, I should like to invite you to venture seriously into this promising research area, the study of the neoplastic process in the living mammalian cell.

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by A. T. SANDISON

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PREFACE

This study is based on work carried out during my appointment as Lecturer in the Department of Pathology of the University of Glasgow, and submitted for the degree of M.D. of the University of St. Andrews. I am grateful to Professor D. F. Cappell for affording me permission and facilities for the investigation, which placed a considerable burden on the routine postmortem service of the department. I am indebted also to the numerous colleagues who gave me access to their autopsies and in many instances permitted me to remove the gonads and endocrine organs for this study; to the technicians in the autopsy room, histological preparation and photographic departments, and members of the secretarial and library staffs for their cheerful and willing cooperation.

My thanks are also due to Dr. Michael J. Brennan and Dr. Michael B. Shimkin for the interest shown in my work.

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An Autopsy Study of the Adult Human Breast: With Special Reference to Proliferative Epithelial Changes of Importance in the Pathology of the Breast [1]

by

A. T. SANDISON, *M.D., B.Sc., Senior Lecturer in Pathology, University of Glasgow, Honorary Consultant Pathologist, Western Infirmary, Glasgow, Scotland*

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[1] Received or publication May 19, 1961; revised September 26, 1961.

Die Geschwülste der weiblichen Brustdrüse sind so oft und so frühzeitig schon gegenstand eifriger histologischer Forschung gewesen, dass man die Brustdrüse in diesem Sinne nicht unpassend die Amme der pathologischen Gewebelehre nennen konnte.

—Eduard Rindfleisch, 1875

. . . Progress is directly proportional to the closeness of the relation of the inquirer and the facts. Now facts, unfortunately are not the natural diet of the mind. They are laborious and often undignified to collect . . .. How different from the noble, shapely, and above all well behaved, conceptions of the mind, which are so manifestly of a higher order of reality.

—Wilfred Trotter, 1935

INTRODUCTION

The epigram of Rindfleisch quoted on the opposite page is probably still as true today as it was when originally penned in 1875. Probably no other neoplasm has been so extensively investigated as mammary carcinoma, and yet it cannot be claimed that the pathology of this tumor has been fully elucidated nor is it true that any great progress has resulted from the data accumulated or from the therapeutic measures thereby suggested. Much remains to be learned about the breast and its pathology, and the present study is an attempt to remedy one obvious deficiency in our knowledge: the lack of reports on the structure of the breasts of persons dying at different ages and from different disease processes.

Over a 5-year period I examined the breasts at consecutive hospital autopsies. While the breasts examined do not fulfill the criterion of a random sample of the general population, it is difficult to conceive how this could ever be practically achieved, so that the autopsy room of a large general hospital probably affords the most realistic approach to a random sample likely to be obtained. While I have been more interested in the female breast because of the practical implications of its greater liability to change, I have also made a study of the male mammary gland and present a summary of the more significant observations.

Because of the large volume of technical work required, I have often been restricted to the histological examination of only one or two selected blocks from each breast. My findings must therefore be regarded as minimal and it is quite possible that the actual incidence of some phenomena is considerably greater than I have been able to demonstrate. Nevertheless, I have been surprised to find that some proliferative lesions of considerable significance are present in many more women, dying of diseases other than primary mammary malignancy, than is generally appreciated.

In this work I have also given comparative figures based on a study of surgically removed specimens of breast, from 1,010 females and 19 males, received in the Pathology Department of the Western Infirmary, Glasgow, over the quinquennium 1950–54 (1958).

HISTORICAL REVIEW

It has been claimed that carcinoma of the breast was known to the ancient Egyptians (Lewison 1955, Loeser 1956) because of certain references to the breast in the Edwin Smith Surgical Papyrus. The Ebers Papyrus, however, contains no alleged references to breast carcinoma and I am informed by Warren R. Dawson (1956) that in his opinion no firm claim can be made that mammary carcinoma was recognized in ancient

Egypt. No evidence of breast cancer was detected in Egyptian mummies (Sigerist 1951), although Ruffer (1911) and others made some examinations of mammary tissue.

The case of Queen Atossa of Persia, wife of Darius, daughter of Cyrus, and mother of Xerxes, often quoted from Herodotus as an example of mammary carcinoma in ancient times was probably really one of inflammatory mastitis (Sandison 1959). Breast carcinoma was, however, certainly known to the Greek physicians of Hippocratic times. Quite apart from references in the Corpus Hippocraticum, votive offerings have been found depicting the breasts and tumor masses [one is figured by La Roe (1947) from the Meyer-Steinegg collection]. Celsus in early Roman times advocated the removal of the breast only in early cancers and Galen only if the tumor was an accessible one. Leonides of Alexandria operated on the breast and was the first to describe nipple retraction as a diagnostic sign.

In medieval times Paré (1510–90) excised easily removable tumors with a margin of normal tissue but in other cases adopted a conservative regime. He is said to have recognized the relationship between the original tumor and axillary extension. Severinus of Salerno (1580–1656) was one of the first surgeons to remove enlarged axillary lymph nodes with the breast tumor and seems to have distinguished between benign and malignant tumors of the organ. Le Dran (1685–1770) believed that cancer spread by the lymphatics to the regional nodes and gave a poorer prognosis when the axillary nodes were involved. Petit (1674–1750) was the first to realize the importance of dissection in continuity.

Later surgeons, *e.g.*, Monro, Velpeau, Boyer, Scarpa, Mayo, etc., emphasized the inevitability of high recurrence rates. Velpeau (1856) wrote a practical treatise on diseases of the breast, but his classification is devoid of histological evidence. In fact, he was largely skeptical of the value of the microscope. The great advances in morbid anatomy and histology made by Virchow and his school soon put knowledge of mammary pathology on a sound footing. Hunter and Paget had previously added further information on the surgical pathology of the breast.

By 1867 Moore had postulated the principles of radical operation except for excision of the pectoral muscles. Halsted (1890–91) devised the modern operation of radical mastectomy, which remains substantially the same at the present time, and he pointed out the danger of delayed diagnosis.

More recently further attempts have been made to utilize our knowledge of the endocrine dependence of some mammary carcinomas (Currie 1958, Bonser and Jull 1960). Beatson (1896) showed that some patients were greatly improved temporarily by bilateral oophorectomy, and Huggins and Dao (1953) by adrenalectomy in advanced mammary carcinoma. Luft and Olivecrona (1955) have taken this concept one step further and advocated surgical hypophysectomy. This operation is however technically difficult and Forrest *et al.* (1956, 1958, 1959) and others have produced destruction of the hypophysis by the insertion of radioactive material. Some of these cases are included in the present series of autopsies.

Cheatle and Cutler (1931) published an extremely valuable monograph on the histology of mammary tumors, while Muir (1935, 1939, 1941) also wrote important papers on the evolution of carcinoma and on Paget's disease. More recently Edith Dawson published a series of histological articles (1932, 1933, 1934–35, 1937, 1954, and 1958) of the greatest value. Haagensen's great monograph (1956) is also indispensable.

Lane-Claypon (1924, 1926, 1928) made statistical studies of the age distribution of carcinoma, and other official statistical studies have shown a real increase in the prevalence of breast cancer, quite apart from its increase due to general aging of the population. Bonser (1945) showed that in mice mammary carcinoma arises multifocally. Burrows (1935) and others showed that administration of estrogen hormones may produce neoplasms in animal breasts. It has since been suggested that estrogens produce hyperplastic cystic change and that retention of secretion thereby may finally provoke neoplasia by some chemical stimulus.

Despite the production of strains of high tumor incidence in mice by close inbreeding, the influence of heredity in human breast neoplasia is not clear and so-called "breast cancer families" are the exception. Morse (1951) has discussed the hereditary aspect of breast cancer in mother and daughter.

The statistical studies of Lane-Claypon showed that mammary carcinoma is more liable to occur in single than in married women and in women of low rather than high parity. Although this premise is not accepted by all authorities, it seems probable that failure to lactate or premature weaning may be factors in the genesis of mammary carcinoma.

The relationship between cystic hyperplasia and carcinoma has been widely discussed by Keynes (1923–24), Cheatle and Cutler (1931), Muir (1941), Dawson (1934–35 and 1943), Foote and Stewart (1945), and Willis (1960) among many other writers, and it seems certain that a significant proportion of cancerous breasts shows significant antecedent hyperplasia. Multifocal carcinoma may arise in both breasts and when carcinoma develops in one of bilaterally hyperplastic breasts the second breast may well be regarded with suspicion and removed by local operation. On the other hand, it is not disputed that some breast cancers are of localized origin and unaccompanied by significant changes.

It is therefore important to try to estimate the frequency of mammary epithelial hyperplasia in the general population. This cannot readily be done, so that our observations are limited to postmortem findings in women. This was done by Frantz *et al.* (1951) who examined the breasts from 225 autopsies and by Franzas (1936) from 100 autopsies. No larger series is known to have been published and I now present my findings in the cases of 800 females and 500 males autopsied in the Western Infirmary of Glasgow.

MATERIAL, METHODS, AND PRESENTATION OF RESULTS

The cases examined were taken from the day-to-day postmortem practice of the Western Infirmary, Glasgow, over a 5-year period; 800 female and 500 male cases were investigated. The cases were unselected and consecutive, subject only to my availability for the removal of material, *i.e.*, during my vacations the breasts were not examined. In the first 525 female cases the gonads and pituitary were not routinely examined, but in the last 275 an attempt was made to include these whenever possible; often other endocrine organs were also examined. Five hundred males were investigated and their testes were also examined when gynecomastia was suspected.

When a postmortem examination was to be made, the body was examined before dissection. Notes were made of the general habitus of the patient, of the size, type, position and appearance of the breasts and, where pertinent, observations on the areola and nipples. Significant changes in the distribution of body hair and external genitalia were also recorded. Included in the protocols were the major morbid anatomical findings which were corrected later, if necessary, when the histological examination of the viscera had been completed by the pathologist performing the autopsy. Particular note was made of any patient showing hyperostosis cranii so that the incidence of breast changes in this condition could be determined.

The breasts of male subjects were entirely removed and fixed before slicing. One block from each slide, showing the maximal area of breast tissue, was then processed, usually by a double-embedding method because of the technical difficulties involved in cutting material which included skin, fibrous tissue, and fat.

In the female, after turning the pectoral flaps, both breasts were repeatedly sliced from behind; any macroscopic abnormality and the relative amounts of fibrous and fatty tissue were recorded. When macroscopic abnormality was detected several blocks of tissue were processed, and at least one large block of breast tissue was taken from the others for histological examination, after a double-embedding technique.

When the ovaries or testes were included in the examination, the entire organs were fixed before the material was sliced and selected for microscopic examination. The pituitaries were fixed *en bloc,* bisected horizontally, and both halves were examined.

In all cases preliminary fixation was carried out in 10 percent formal-saline and after selection of blocks these were adequately fixed in formal-sublimate (Lendrum 1941) before processing.

Hemalum and eosin staining was the routine method used but more special methods were occasionally applied, *e.g.*, Weigert's stain for periductal elastic tissue; Lendrum's Carbacid-fuchsin method (1945) for apocrine granules; Lendrum's lissamine red method (1951), and phosphotungstic acid-hematoxylin for myoepithelium, as well as the conventional trichrome staining methods.

For examination of the pituitary, Pearse's method (1949, 1950) was used, in addition to older methods, while phosphotungstic acid-hematoxylin was valuable for the demonstration of the alpha cells.

Brief summaries of my findings in the 800 female autopsies appear in my thesis which is lodged in the Library of the University of St. Andrews (1957). Some findings were further briefly summarized in the sections on epitheliosis, papillomatosis, carcinoma, and sclerosing adenosis, and in other smaller sections dealing with the appearance of the breast in certain general disease processes. These may also be consulted in my thesis. An addendum is included here which briefly lists the cases and indicates the type of autopsy population studied.

In most instances pertinent analyses of findings are presented as tables, often with subdivision into quinquennial groups. Cases under age 20 are excluded from this series as they belong more properly to a study of the development of the breast from the neonatal period to maturity.

Pertinent literature on each subject considered is briefly reviewed, the results of my analyses are given, and the findings are then briefly summarized at the end of each section.

I have directed most of my attention to the proliferative changes in the breast as I consider these to be very important. Other phenomena, such as the presence of cysts, ectasia, apocrine metaplasia, etc., are dealt with simply as these have been exhaustively studied in the past.

In the male series, no case histories were compiled as in many instances nothing of note was discovered. Here again, most of the emphasis is on proliferative changes, while other phenomena are only briefly considered.

TYPES OF BREAST

At the onset of this study it seemed worthwhile to note the types of breast in the protocols of my cases. However, this was not easy because of the lack of suitable criteria for a system of classification, and because the supine position at autopsy tends to flatten the breast over the chest wall. Most classifications of breast type are based on the erect posture which reveals any pendulous tendency.

A working classification for typing the breast is given by Geschickter (1945) and on this I have based my classification. Some interesting data on individual and racial breast shape, size, etc., are given by Stratz (1908, 1923), Rhiel (1927), Ploss and Bartels (1935), La Roe (1947), and Siemens (1952). The latter author made a special study of the pendulous breast, with reference to the breast form in art. For information on the development of the adolescent breast, the paper of Reynolds and Wines (1948) and the monograph of Tanner (1955) may be consulted.

The classification I have adopted is based on three main types: the "atrophic," the "fatty," and the relatively normal, "virginal" breast. The atrophic breast is small and fibrous, lacks a fatty envelope, and often has a lax, wrinkled skin covering. The fatty breast is large, rounded, and shows more or less disappearance of the fibrous parenchyma and its replacement by fatty tissue; the covering skin is usually smooth. The less severe form I have classified "adipose" and the more severe "obese." In this classification the virginal breast would be placed between the atrophic and adipose. It is rounded, smooth, and has a fatty envelope around a well-preserved fibrous parenchyma. If small and rather flat, I have termed it hypovirginal and if large, hemispherical, or conical, "hypervirginal." Since these terms are empirical and subjective, I have not stressed this classification, and for analysis of microscopic findings I have frequently chosen to subdivide simply into the criteria of "fibrous" and "fatty." The extremely pendulous breasts shown by Siemens (*die Hängebrust*) have rarely been seen in this work. Perhaps the reduction in family size, limitation of lactation, and the wider use of the brassière since Victorian and Edwardian times have led to this change.

For valuable histological studies covering the development of the normal breast, reference should be made to Dawson (1934–35), Bässler (1958 *a* and *b*), and Ingleby and Gershon-Cohen (1960). These include development from the embryo to the mature woman and also the changes resulting from pregnancy and lactation. A shorter account is given by Geschickter (1945); for the surgical anatomy of the breast *see* Handley (1955) and for physiology *see* Trimble (1955).

Before discussing results, I would point out the prevalence of the concept among surgeons and pathologists that the breast is an essentially fatty organ. This is no doubt the result of examination of a large number of obese breasts which have been removed by radical operation. The breast however of the young woman is an irregular cone of fibrous tissue investing the epithelial-lined duct tree and covered by a fatty envelope. Breast stroma has been studied morphologically and histochemically by Ihnen and Perez-Tamayo (1953). With involution, the breast may atrophy without fatty infiltration and remain tough and fibrous, or it may atrophy with fatty infiltration. When this change is extreme, it may be difficult to detect remaining fibrous tissue. For an interesting hypothesis that fibrosis of the mammary stroma is hormonal in origin, *see* Vassar and Culling (1959). The inexperienced surgeon incising the normal breast of a young woman may marvel at the early age at which "chronic mastitis" begins, when what he is actually seeing is the fibrous breast that is normal for this period of life.

Results

The gross breast types are shown in tables 1 and 2. The over-all incidence from 21 to 90 years indicates a predominance of the fibrous (54.25%) over the fatty group (45.75%). Up to age 45 the great majority of breasts are fibrous and from 56 to 70 years fatty breasts are more common. In women who survive to age 70, the breasts at autopsy are more likely to be of the atrophic fibrous type than fatty. This, however, may possibly only reflect the likelihood that the spare, elderly woman may survive longer than her more obese sister.

TABLE 1.—Gross breast types

Age group	Atrophic	Adipose	Obese	Virginal	Hyper-virginal	Hypo-virginal	Pregnancy and lactation	Total
21–25	—	—	—	5	1	4	3	13
26–30	1	1	—	7	1	1	1	12
31–35	8	3	2	13	1	14	3	44
36–40	6	3	3	15	1	4	1	33
41–45	23	9	2	9	1	1	—	45
46–50	25	17	3	7	4	2	—	58
51–55	46	33	11	6	2	—	—	98
56–60	26	42	17	5	2	2	—	94
61–65	51	51	32	2	1	1	—	138
66–70	40	51	22	—	1	—	—	114
71–75	45	36	10	1	5	—	—	97
76–80	22	9	4	—	1	1	—	37
81–85	8	4	—	—	—	—	—	12
86–90	4	1	—	—	—	—	—	5
21–90	305	260	106	70	22	30	8	800

In the more detailed classification, the following characteristics emerge: The breasts of women under 30 are mostly variants of the virginal type (although 1 of adipose and 1 of atrophic type are noted); between 30 and 40, the virginal types still predominate, but a significant proportion already show atrophy and some must be classified as adipose or even obese. In the following periods the number of virginal types rapidly decrease, although odd cases of this character are seen even at advanced ages. Elderly women occasionally maintain their breast contours without excessive atrophy or fatty infiltration, but after age 80 all the breasts seen are atrophic. In the middle period of life the adipose or obese groups predominate.

Of the 800 women studied, 8 showed change resulting from lactation or pregnancy, 305 showed atrophy, 260 were adipose, 106 were obese, and 70 were virginal in type.

TABLE 2.—Breast types

Age group	Fibrous group		Fatty group		Total
	Number	Percent	Number	Percent	
21–25	13	100	—	—	13
26–30	11	91. 6	1	8. 4	12
31–35	39	88. 7	5	11. 3	44
36–40	27	81. 8	6	11. 2	33
41–45	34	75. 5	11	24. 5	45
46–50	38	65. 5	20	34. 5	58
51–55	54	55. 1	44	44. 9	98
56–60	35	37. 2	59	62. 8	94
61–65	55	39. 8	83	60. 2	138
66–70	41	35. 9	73	64. 1	114
71–75	51	52. 2	46	47. 4	97
76–80	24	64. 8	13	35. 2	37
81–85	8	66. 6	4	33. 4	12
86–90	4	80. 0	1	20. 0	5
21–90	434	54. 3	366	45. 8	800

No case of gross hypertrophy, amazia, athelia, polymazia, or polythelia was seen, nor were supernumerary breasts in other sites seen in either sex. However, in one 66-year-old woman with large breasts, one was half as large as the other; the smaller was fatty and the larger fibrofatty on section.

Summary

In younger women the breast is usually fibrous, in many middle-aged women it is fatty, while in those surviving until the ninth decade it is always fibrous. Breasts of normal virginal appearance are occasionally seen in old women but the virginal appearance of the younger breast usually changes to the atrophic or fatty type.

THE "NORMAL" BREAST

Since the breast responds rhythmically to the hormonal cycles induced by the pituitary and ovary and is probably never at rest, it is not surprising that the "normal" breast is something difficult to define and rarely seen. In this study I have included under the title "Adenosis" all cases showing well-formed active lobules, and it is fairly certain that before the menopause these are in fact manifestations of the normal breast. In table 3, I have chosen the arbitrary age of 45 and indicated all adenosis (62 cases) below this age as physiological. The histological diagnosis of normal breast without adenosis was made in 7 cases between the ages of 22 and 40. Eight more cases are included under the heading of pregnancy or lactation (all between the ages of 23 and 38). These we may assume are normal variants, since pregnancy and lactation are physiological phenomena. This brings the total of normal breasts to 77 out of 147 cases under 45 years of age, an incidence of 52.3 percent. If we consider

TABLE 3.—Adenosis and proliferative lesion in the "normal" breast

Age group	Physiological adenosis	All other proliferative lesions	Total cases	Percent of cases with proliferation
21–25	5	—	13	—
26–30	7	2	12	16. 6
31–35	21	7	44	15. 9
36–40	11	7	33	21. 2
41–45	18	17	45	37. 7
46–50	—	27	58	46. 5
51–55	—	47	98	47. 9
56–60	—	42	94	44. 7
61–65	—	61	138	44. 2
66–70	—	48	114	42. 1
71–75	—	39	97	42. 3
76–80	—	16	37	43. 2
81–85	—	7	12	58. 3
86–90	—	—	5	—
21–90	62	320	800	40. 0

the over-all-incidence age 21 to 90 years, the incidence of "normal" breasts falls to 9.6 percent. This however is an unrealistic figure as it is probable that "atrophic" is indeed the normal state of postmenopausal breasts. (*See* section "Atrophy, Duct Ectasia, and Cyst Formation.") If we consider all cases over 45, we find that 363 of 653, 55.6 percent, are atrophied, presumably as a result of involution and these might be regarded as "normal" for this group. Thus, our "normal" groups over the entire age range (440 of 800 cases) represent an over-all incidence of 55 percent.

ATROPHY, DUCT ECTASIA, AND CYST FORMATION

In this section my main concern is to establish the number of atrophic breasts in my series in contrast to those showing continuing proliferative change. I include some figures on the total incidence of duct ectasia and cyst formation in all specimens, and also an estimate of the number of atrophic breasts showing ectasia or cyst formation as the only feature of note.

It is generally agreed that, with involution of the breast after the menopause, atrophy takes place in a considerable number of cases. This is briefly discussed by Schultz (1933) who correlates it with cystic duct ectasia and elastosis (a subject that I will discuss in a later section). Schultz talks of "Zysticher Erweiterung der Ausfuhrungs-gänge" and I sometimes find it difficult to differentiate between duct ectasia and cyst formation in sections. For that reason I consider these phenomena together in this part of my analysis. Haagensen (1951, 1956), in an interesting discussion on mammary-duct ectasia, deals especially with the more gross forms of ectasia which lead to surgical intervention. Similar appearances of lesser degree may be seen in the postmortem breast; the ducts are dilated, often thickened, and contain a bluish-green grumous retained secretion which is largely amorphous on microscopic examination. Indeed, this material may discharge spontaneously from the nipple and, in my experience, may lead the clinician to believe that a papilloma of the breast is present. Unnecessary mastectomies are sometimes performed. I have found that examination of nipple discharge by Papanicolaou's method is an invaluable diagnostic aid (Walker and Sandison 1958).

Frantz *et al.* (1951) stated that duct ectasia occurred in about 25 percent of their 225 postmortem cases. Of the 52 with ectasia, 96.3 percent were postmenopausal and 20.4 percent showed cyst formation also.

Much has been written about cyst formation in the breast and this subject in relation to epitheliosis (hyperplastic cystic disease) is discussed later in this monograph. A full discussion may be found in Schultz (1933) under the title of "Die Zystenmamma." The recent work of Ingleby and Gershon-Cohen (1960) raises many points of interest also.

The detailed monograph by Semb (1928), which has a very exhaustive bibliography, surveys critically the earlier work of Astley Cooper, Velpeau, Reclus, Brissaud, and Schimmelbusch. Bloodgood (1921) devoted much time and thought to the problem of cystic disease in his work. He elaborated a classification of macroscopic mastopathy, recognizing 8 different types. This is now of historic interest only and of little practical value, but an important point made by Bloodgood was the essentially benign nature of the "blue domed" cyst that now bears his name.

Turning now to the views of more recent writers, Cappell (1958) reiterates Muir's opinion on cyst formation. Muir believes that cyst formation depends to some extent on duct dilatation (and later dilatation of acini also) from abnormal secretion. There is usually fibrosis also, especially around the acini. I have shown (1956) that remarkable dilatation of the breast ducts may occur in the neonatal period in a considerable proportion of infants, as the result of much "hexen-milch" secretion produced by the transplacental passage of maternal hormone. As the circulating hormone decreases, the epithelium begins to involute and the ducts appear ectatic or even almost cystic and are lined by a simple flattened epithelium.

Foote and Stewart (1945) showed in their examination of 300 cancerous and 200 noncancerous breasts that cysts were more common in noncancerous breasts (53 against 27% of cases) and most frequent in the fifth decade and relatively infrequent in the seventh. They agreed that of the 300 cancers studied only 2 could have arisen in a cyst. They claim that blunt duct adenosis (a term which they coined) may lead to cyst formation in noncancerous breasts while cancerous breasts may tend to become proliferative. They believe this type of adenosis is a proliferation of blunt end ducts, arising at the distal extremity of the duct system, which lacks a myoid investment. There is no doubt that one sees dilated acini (as Muir also described), but whether the neologism of Foote and Stewart is justifiable remains to be seen.

Ingleby (1942), Gershon-Cohen and Ingleby (1952), and Ingleby and Gershon-Cohen (1960) have discussed cyst formation in the breast in relation to normal and pathological proliferations. Ingleby makes the interesting observation that in simple cystic disease the cysts resemble the swellings of the growing end branches of the normal developing breast, but unlike these vary greatly in size and distribution. Ingleby believes that this type of cystic disease is the result of a puberty type of stimulus acting on a breast already undergoing cyclical changes and that many apparent lobules are represented by bunches of cysts. This finding may perhaps be equated with "blunt duct adenosis."

Ingleby separates another variety which she terms "secretory cystic disease." In this form the ducts are dilated with inspissated secretion. The condition may be more or less generalized and accompanied by simple cystic change. This process in the ducts may also be associated with colostrum corpuscle formation. This "secretory cystic disease" is synonymous with the condition which I regard as "ectasia" of the ducts, and I am therefore supported by Ingleby in considering together "ectasia" and simple cyst formation in the present study.

Some reports of postmortem studies should now be mentioned. Semb (1928) examined 64 breasts from 32 women at autopsy (6 were pregnant or lactating) and claimed that neither fibroadenomatosis cystica simplex nor fibroadenomatosis cystica papillomatosa could be found, but that fibroadenomatosis simplex microcystica was present in 1 case. This incidence of 3.8 percent in 26 nonpregnant cases seems very low. Semb cites Tietze as having found cysts in 4 of 17 women aged over 40 (23.5%); Goens to have found cysts in 33.3 percent of 30 women over 40, and Hahn to have found cysts in 11 of 48 younger women, *i.e.*, 22.9 percent.

Lindgred (1936–37) studied 120 breasts from 60 autopsies of women aged 35 to 75 and found cystic change and duct ectasia to be common. He noted 4 blue domed cysts and 10 other macroscopic cysts; cyst formation was usually bilateral.

Frantz *et al.* (1951), in a careful study of postmortem breasts from 225 autopsies on women aged 13 to 88, found ectasia of ducts in 54 cases (24.0%) and gross cystic disease in 42 (18.6%); 33 showed microcysts only, the ages ranging from the second to the ninth decade. Two of these were associated with duct ectasia. Frantz *et al.* rightly interpret these figures as showing a low incidence of chronic cystic disease in the surgical sense.

Results

If we consider first all cases showing macro- or microcysts or appreciable ectasia of ducts, we find 574 out of 800 cases, an over-all incidence of 71.7 percent. The maximum incidence occurred in the periods 76 to 80 and 81 to 85 (83%), but between the ages of 46 and 75 more than 70 percent showed some degree of ectasia or cysts. In the fourth decade more than 60 percent were affected. By contrast, from 21 to 25 years only 15.4 percent of cases showed cysts or ectasia, and only 1 of 5, or 20 percent, in the period 86 to 90.

If we view those cases in which the primary histological diagnosis was macroscopically appreciable duct ectasia or cysts in an atrophic breast parenchyma, we find that this occurred in only 87 of the 800 cases, an over-all incidence of 10.9 percent—slightly higher in fatty (11.5%) than in fibrous breasts (10.4%). The maximum incidence (17.5%) was in the period 66 to 70 and the incidence from 81 to 85 was 25 percent, although here the number of cases was few. The over-all incidence was higher in the third decade than in the fourth and rose again in the fifth. The difference in incidence in fibrous and fatty breasts in the different quinquennial age periods showed a haphazard pattern and no really significant differences were observed.

Turning now to cases in which the primary histological diagnosis was atrophy without macroscopic cyst formation or ectasia, we find that the over-all incidence was 302 out of 800 cases, *i.e.*, 37.8 percent, with a rather higher proportion in fatty (42.3%) than in fibrous breasts (33.9%). The incidence of atrophy, as might be expected, rose with increasing years from 8.3 percent in the period 26 to 30 to 54.1 percent in the period 76 to 80. (From 81 to 85 years the figure was 25.0%, but here the numbers are few.) In the 86- to 90-year group all breasts were atrophic (fig. 1). If we divide the cases into fibrous and fatty breasts, it is seen that in women under age 55 atrophy is more frequent in fatty breasts and over 55 in fibrous breasts. Active intralobular fibrosis was noted only in 3.7 percent of all cases.

Finally, if we examine together all cases showing essential atrophy of the breast parenchyma with or without cyst formation or duct ectasia,

Table 4.—Primary histological diagnosis: atrophy

	Breast type					
	Fibrous		Fatty		Total	
Age group	Number of cases	Percent* of all cases	Number of cases	Percent of all cases	Number of cases	Percent of all cases
21–25	—		—		—	
26–30	—		1	100. 0	1	8. 3
31–35	5	12. 8	—		5	11. 4
36–40	4	14. 8	2	33. 3	6	18. 2
41–45	4	11. 8	2	18. 2	6	13. 3
46–50	13	34. 2	10	50. 0	23	39. 8
51–55	20	37. 0	22	50. 0	42	42. 9
56–60	16	45. 7	24	40. 7	40	42. 6
61–65	26	47. 3	35	42. 2	61	44. 2
66–70	17	41. 5	30	41. 1	47	41. 2
71–75	21	41. 2	22	47. 8	43	44. 3
76–80	14	58. 3	6	46. 2	20	54. 1
81–85	3	37. 5	—		3	25. 0
86–90	4	100. 0	1	100. 0	5	100. 0
21–90	147	33. 9	155	42. 3	302	37. 8

* Numbers from which percentiles are derived are given in table 2.

Table 5.—Primary histological diagnosis: ectasia or cysts

	Breast type					
Age group	Fibrous	Percent of all cases	Fatty	Percent of all cases	Total	Percent of all cases
21–25	1	7. 7	—		1	7. 7
26–30	1	9. 1	—		1	8. 3
31–35	—		1	20. 0	1	2. 3
36–40	1	3. 7	—		1	3. 0
41–45	2	5. 9	2	18. 2	4	8. 9
46–50	6	15. 8	2	10. 0	8	13. 8
51–55	2	3. 7	7	15. 9	9	9. 2
56–60	3	8. 6	6	10. 2	9	9. 6
61–65	9	18. 2	7	8. 4	16	11. 6
66–70	9	21. 9	11	15. 1	20	17. 5
71–75	7	13. 7	5	10. 9	12	12. 4
76–80	2	8. 3	—	—	2	5. 4
81–85	2	25. 0	1	25. 0	3	25. 0
86–90	—		—		—	
21–90	45	10. 4	42	11. 5	87	10. 9

TABLE 6.—Primary histological diagnosis: atrophy, ectasia, or cysts

Age group	Breast type: Fibrous — Number of cases	Fibrous — Percent of all cases	Fatty — Number of cases	Fatty — Percent of all cases	Total — Number of cases	Total — Percent of all cases
21–25	1	7. 7	—		1	7. 7
26–30	1	9. 1	1	100. 0	2	16. 6
31–35	5	12. 8	1	20. 0	6	13. 6
36–40	5	18. 5	2	33. 3	7	21. 2
41–45	6	17. 6	4	36. 4	10	22. 2
46–50	19	50. 0	12	60. 0	31	53. 4
51–55	22	40. 7	29	65. 9	51	52. 0
56–60	19	54. 3	30	50. 8	49	52. 1
61–65	35	63. 6	42	50. 6	77	55. 8
66–70	26	63. 9	41	56. 2	67	58. 7
71–75	28	54. 9	27	58. 7	55	56. 7
76–80	16	66. 7	6	46. 2	22	59. 5
81–85	5	62. 5	1	25. 0	6	50. 0
86–90	4	100. 0	1	100. 0	5	100. 0
21–90	192	44. 2	197	53. 8	389	48. 6

TABLE 7.—All breasts showing cysts or ectasia of ducts

Age group	Number of cases	Percent of all cases
21–25	2	15. 4
26–30	7	58. 3
31–35	19	43. 2
36–40	21	63. 6
41–45	30	66. 6
46–50	43	77. 9
51–55	70	71. 4
56–60	74	78. 7
61–65	102	73. 8
66–70	87	76. 3
71–75	77	79. 4
76–80	31	83. 8
81–85	10	83. 3
86–90	1	20. 0
21–90	574	71. 7

nearly half, *i.e.*, 389 of 800 or 48.6 percent, showed atrophy as the essential appearance in the breast. One case so classified in the age group 21 to 25 may probably be regarded as failure to develop lobules (fig. 5), but, this instance apart, atrophy is a feature from 26 to 90 years. The incidence increases with age, and after 45 more than half the cases showed essentially atrophic changes. Once again it is noted that under age 55 atrophy is more common in women with fatty breasts and over 55 in women with fibrous breasts (tables 4, 5, 6, and 7).

Summary

Nearly 50 percent of all breasts examined in this series were essentially atrophic and 37.8 percent of all showed atrophy as the primary histological diagnosis. About 10 percent showed macroscopically evident cysts or duct ectasia in an atrophic parenchyma. The over-all incidence, however, of cysts, microcysts, and duct ectasia was just over 70 percent. Under age 55, atrophy was more common in women with fatty breasts and in those with fibrous breasts over that age. The only postmortem series with which a direct comparison can be made is that of Frantz *et al.* If we add their figures for ectasia, gross cysts, and microcysts the total is 129 of 225 cases, an incidence of 57.3 percent which is significantly smaller than my figure of 71.7 percent. However, the difference is probably the result of subjective differences in interpretation of what constitutes a significant cyst formation or ectasia of ducts. In both series over half the breasts examined showed these changes.

ADENOSIS

I use this term in the sense first defined by Dawson (1933), at the instigation of the late W. F. Harvey, and subsequently elaborated further by her in valuable detail (1934, 1935). It implies a glandular hyperplasia which produces more and larger lobules of normal or exaggerated physiological pattern in the breast parenchyma. It should be emphasized that "adenosis" is generally assumed to have no pathological implication although, as we shall see, some authors misuse the term and include conditions more properly to be regarded as epitheliosis. Adenosis is particularly noted at adolescence, in pregnancy and lactation, and at the menopause, while there is also a recurring cyclical change occurring throughout the menstrual cycle (figs. 2, 3, and 4). Adenosis at adolescence is a slow process. In pregnancy it is extensive and well marked with secretory changes superadded in lactation. After lactation, involution leads to marked regression so that theoretically each pregnancy leads to a new phase of adenosis. My present study, however, shows that involution of lactation is not always complete, and isolated foci of secretory change may be seen in elderly women long after the menopause (fig. 39).

As mentioned previously the term "adenosis" has sometimes been misused: Geschickter (1945) assumes it is synonymous with Schimmelbusch's disease and includes what is called "epitheliosis" by Dawson (*i.e.*, intraductal epithelial hyperplasia), with papillomatosis and adenoma formation also included. There is a tendency in the London medical schools to use the term fibroadenosis as synonymous with cystic disease; this appears to be unnecessary, faulty usage. However, other authors of compendious works, *e.g.*, Saner (1950), use the term correctly to imply lobular enlargement with increase in the number of acini and perhaps hyperplasia of the periacinous fibrous tissue and some round-cell infiltration.

The term "mazoplasia" introduced by Cheatle and Cutler (1931) is virtually synonymous with adenosis as I understand it. These authors implied that "mazoplasia" is a hyperplasia of the pericanalicular and periacinar connective tissue with new formation of ducts and acini, usually accompanied by desquamation of some epithelial cells in the terminal ducts and acini. They believed it is related to no pathological state, except perhaps the formation of fibroadenoma, and almost universal in parous women before the menopause. In brief, Cheatle and Cutler regarded mazoplasia as largely a physiological state, which tends to disappear at the menopause. They commented on the fact that adenosis is most marked in the premenstrual phase.

Dieckmann (1925) described the cyclical changes in the breast with relation to the menstrual cycle and Schultz (1933) reviewed this subject fully. More recently Ingleby (1942) discussed normal and pathological proliferations in the breast, based on morphological studies of the adolescent gland. She emphasized the nonuniformity of development and regression of lobules in the sex cycle. Gershon-Cohen and Ingleby (1952) in a further paper subdivided mazoplasia (which here they equate with adenosis) into two groups, one of which shows hyperplasia of the subepithelial fibrillary zone and the other of the lobular acini proper. Ingleby and Gershon-Cohen (1954) further classified adenosis, describing in some detail the cyclical changes in the lobule. In the postmenstrual phase budding of ductules occurs to form new lobules; the formative bud appears as a proliferation of cells at some point on the wall of a prelobular duct. A lumen is formed, the duct continues to proliferate and produces new ductules which also grow and branch until a new lobule is developed. The epithelial and basal cells are at first hyperchromatic, but as the menstrual cycle proceeds they become larger and paler; the myoepithelium is formed by the separation of blunt processes from the basal cells which later elongate and become striated. Some days before menstruation, protein secretion appears in the ducts and the epithelial cells may vacuolate; eventually the epithelium is shed into the lumen and the stroma becomes loose and edematous, presumably as part of the retention of fluid occurring at this time, when the breasts often become noticeably enlarged. Ingleby and Gershon-Cohen (1960) have summarized their current views on adenosis based on observations using the slicer technique. Ingleby (1949) measured the breast volume in normal women students and showed that there is a midmenstrual rise, probably coincident with ovulation, followed by a drop, and then a further rise to a peak in the week before menstruation. With this edema in the lobular fibrillary connective tissue there is also an infiltration of mononuclear cells, most of which resemble lymphocytes. Ingleby and Gershon-Cohen (1954) further divide adenosis into four groups. In type A there is lobular hyperplasia with very large lobules or numerous closely set smaller lobules, while in type B there is incomplete lobular development with dilated ductules. Type C is not very clearly defined but is said to occur near carcinomatous areas in the breasts of young women. Type D is synonymous with "sclerosing adenosis" which these authors prefer to name "myoid sclerosis" because of their belief that the formations concerned are largely myoepithelial. (I will deal at length with sclerosing adenosis later.) The views of Ingleby and Gershon-Cohen on the significance of adenosis are not quite clear, but they apparently regard type C as dangerous and possibly precancerous. It is unfortunate that they are unable to define this type more clearly, but it appears that it is regarded as being deficient in myoepithelial elements. From their description I believe that it corresponds more closely to what I have considered to be intra-acinar epitheliosis rather than a form of adenosis. It seems that Dawson (1954) also finds this group rather incomprehensible.

Records of the frequency of adenosis in postmortem breast studies are scanty. Semb (1928) found only 1 case with adenosis in 32 autopsies, excluding 6 others who were either pregnant or lactating. Keynes (1923–24) in a series of 116 autopsies noted that 57 of these were normal and 59 showed "inflammatory lesions," *e.g.*, dilatation of ducts and acini, accumulation of products of epithelial activity, infiltration by lymphocytes, fibrosis, and changes in the epithelium. It is not possible from his figures to determine the incidence of adenosis. Lindgred (1936–37) studied 120 breasts from 60 autopsies and found mazoplasia in 22 glands, an incidence of about 18 percent. Frantz *et al.* (1951), in a detailed study of the female breast in 225 autopsies, found adenosis in only 14 cases, an incidence of 6.2 percent, but in 7 of 42 cases showing cystic change, an incidence of 16.7 percent. Franzas (1936—cited by Frantz *et al.* in an additional reference to their paper after publication) is said to have corroborated their findings in a series of 100 postmortem cases, but no specific reference is made to adenosis.

Results

Tables 8 and 9 show that the over-all incidence of adenosis as the only proliferative finding in the breast was 16.7 percent, a figure corresponding with that of Lindgred. As expected, this figure is largely

Table 8.—Pure adenosis*

	Breast type							
	Atrophic		Adipose-obese		Virginal group		Total	
Age group	Number	Percent	Number	Percent	Number	Percent	Number	Percent
21–25	—		—	—	5	45. 4	5	38. 5
26–30	—	—	—	—	5	50. 0	5	41. 7
31–35	4	44. 4	2	40. 0	15	51. 7	21	47. 7
36–40	1	16. 6	3	50. 0	7	33. 3	11	33. 3
41–45	10	43. 5	6	54. 5	3	27. 3	19	42. 2
46–50	6	24. 0	4	20. 0	4	30. 7	14	24. 1
51–55	8	17. 4	7	15. 9	1	12. 5	16	16. 3
56–60	6	23. 1	8	13. 5	—	—	14	14. 9
61–65	3	5. 9	8	9. 6	—	—	11	7. 9
66–70	6	15. 0	5	6. 8	—	—	11	9. 6
71–75	3	6. 7	1	2. 2	—	—	4	4. 1
76–80	1	4. 5	—	—	—	—	1	2. 7
81–85	2	25. 0	—	—	—	—	2	1. 7
86–90	—	—	—	—	—	—	—	—
21–90	50	16. 3	44	12. 0	40	32. 0	134	16. 7

*This does not include an adenosis of pregnancy in the quinquennium 31 to 35.

TABLE 9.—All cases with adenosis

	Breast type							
	Atrophic		Adipose-obese		Virginal group		Total	
Age group	Number	Percent	Number	Percent	Number	Percent	Number	Percent
21–25	—	—	—	—	5	45.4	5	38.5
26–30	—	—	—	—	6	60.0	6	50.0
31–35	5	55.5	3	60.0	18	62.1	26	59.1
36–40	1	16.6	3	50.0	9	42.7	13	39.4
41–45	15	65.2	7	63.6	4	36.3	26	57.8
46–50	8	32.0	5	25.0	5	38.5	18	31.0
51–55	11	23.9	10	22.8	3	37.5	24	24.5
56–60	8	38.8	12	20.3	1	11.1	21	22.3
61–65	4	7.8	11	13.2	—	—	15	10.8
66–70	6	15.0	7	9.6	—	—	13	11.4
71–75	5	11.1	3	6.5	—	—	8	8.2
76–80	1	4.5	1	7.7	—	—	2	5.4
81–85	3	37.5	—	—	—	—	3	25.0
86–90	—	—	—	—	—	—	—	
21–90	67	37.2	62	34.4	51	37.3	180	

comprised of cases occurring up to age 60 in 105 out of 397 cases, while only 29 of 403 were noted after this age, when ovarian activity probably had ceased. Further, the maximum incidence occurred in the quinquennium 31 to 35 (47.7%) while there was a sharp drop from 42.2 percent in the quinquennium 41 to 45 to 24.1 percent in the following quinquennium. It is probable that there is a relationship here to the menopause, which Dawson (1943) has shown to occur most frequently from age 49 to 51 years. As expected also, pure adenosis was most frequently seen in the virginal breast (32.0% of all cases). It appeared least frequently in the adipose-obese group overall, though between the ages of 31 and 45 the relatively few breasts which were adipose or obese showed adenosis more frequently than other types.

When adenosis is considered not as an isolated condition, but either alone or accompanying other proliferative changes, the over-all incidence in the two types of fibrous breast, atrophic or virginal, was very similar while the incidence in fatty breasts was only slightly lower. It would seem therefore that when all instances of adenosis are considered the over-all incidence is not affected by the breast parenchymal type. It was more common up to age 60, *i.e.*, 139 of 397 cases as against 41 of 403 after that age. Adenosis was present at times in conjunction with all the other changes noted in the breast, and this will be referred to when the other proliferative changes are considered.

Regarding the ovaries, we may say in general that when adenosis and sclerosing adenosis are considered together, there appears to be a relationship between the percentile incidence of cases showing adenosis and the degree of stromal cellularity of the ovary, *i.e.*, 34.7 percent of women with ovarian stromal cellularity + + + showed adenosis while the incidence was 29.3 and 21.1 percent, respectively, for women with stromal cellularity + + and +. It is not surprising that of 31 women between the ages of 21 and 55 who showed either follicular activity or luteal tissue, 20 showed adenosis or sclerosing adenosis, an incidence of 64.5 percent, and of 18 women between the ages of 31 and 85 with prominent interstitial cells at the hilum of the ovary, 10 showed adenosis or sclerosing adenosis, an incidence of 55.5 percent.

Summary

Adenosis as the sole proliferative lesion occurred in 16.7 percent of all cases, the majority being under age 60 and the maximum in the quinquennium 31 to 35 years—the virginal type of breast was most frequently affected. When adenosis was regarded broadly with or without accompanying changes, it occurred without significant difference in fibrous and fatty breasts and much more frequently before the age of 60, the maximum being in the quinquennium 31 to 35 years when presumably the effects of past pregnancies and lactations are having their maximal effect.

Finally there may be a relationship between the degree of cellularity of the ovarian stroma and the incidence of adenosis. There was a high proportion in women with persisting follicular or luteal activity.

I have seen nothing in my studies which would lead me to disagree with the opinions of Cheatle and Cutler or Dawson that adenosis has probably no causal relationship with any of the more significant pathological changes such as epitheliosis or the development of carcinoma.

SCLEROSING ADENOSIS

This interesting and important form of mammary adenosis has also been termed fibrosing adenosis and myoid sclerosis. It is of great significance to the practicing surgical pathologist, who has to perform frozen-section examinations of the breast in the operating theater, because of the possibility of confusing this condition with scirrhous carcinoma.

I have published a survey of 1,010 female surgical breast specimens received in the Pathology Department of the Western Infirmary, Glasgow, during the years 1950–54. In it special attention was given to sclerosing adenosis, and I will refer to these findings later (1958).

At this point it would seem important to outline the salient features of sclerosing adenosis. It is a condition occurring most frequently in younger women, possibly due to some temporary hormonal imbalance, and producing a firm, mobile nodular or discoid mass in the breast. To the naked eye it appears as a grayish-white nodular mass with hyperplastic lobular areas sometimes associated with cysts. Histological examination confirms the lobular nature of the lesion—a most important diagnostic point. The cytological character varies with the phase at which the condition is seen. There is an early florid phase which regresses and then goes on through an intermediate or actively sclerosing stage to a final fibrosed lesion. In the florid phase there is epithelial and myoepithelial hyperplasia in the lobules with the formation of narrow tubules or solid cords of cells. The stroma is delicate, the cells active, and mitotic figures may be present. Later there is proliferation of connective-tissue stroma as the lesion regresses till finally the epithelial cords are attenuated and compressed by fairly dense hyaline connective tissue. This appearance is strongly suggestive of scirrhous spheroidal cell carcinoma of the breast, and may be readily so diagnosed if viewed under the high power of the microscope. It cannot be too strongly emphasized that in the study of breast tissue, especially in frozen sections, great weight must be given to the impression obtained under very low magnification with a scanning objective, *e.g.*, ×4, and preferably a wide-angle eyepiece, which gives a well-illuminated large field.

The existence of sclerosing adenosis was not recognized by the older authorities, *e.g.*, Muir (1941) or Cheatle and Cutler (1931). A brief description of this condition is however given by Ewing (1940), who points out that this condition more than any other in the breast is likely to be mistaken for carcinoma. This warning has been reiterated by Boyd (1947) while Foot (1945) mentions "diffuse fibrosing adenomatosis" but states that its presence does not alter the prognosis of fibrocystic disease. Saner (1950) regards the condition as a form of hyperplastic cystic disease which may be difficult to diagnose on immediate theater examination.

Mulligan (1951) gives a valuable summary of his views on sclerosing adenosis: He mentions that it is more commonly seen as an incidental microscopic finding than as a discrete mass which is rarely large. The lesion, which is most commonly found between the ages of 20 and 40, begins within the lobules or in terminal ductules, and in the early stage shows considerable proliferative activity with formation of narrow tubular spaces or solid sinus cords of cells. There may be some variation in cell size and shape and occasional normal mitoses. At this stage the stroma is very delicate. Throughout the evolution of the lesion a distinct lobular pattern persists. In the later phase the florid appearance of proliferation gives way to an overgrowth of connective tissue, which later shows focal hyalinization and mature fibrocytes. This connective tissue tends to compress the attenuated cords of epithelial cells. The sclerosing effect may be irregular and is often most severe at the lobule periphery. A bizarre pattern may thus be produced, distinctly reminiscent of scirrhous carcinoma.

Willis (1960) also stresses the collapse, atrophy, and distortion of the epithelial elements with stromal fibrosis and states that there is a superficial resemblance to infiltrating scirrhous carcinoma. He emphasizes that mistaken diagnoses may be made, especially in frozen sections, and that the only safeguard is experience in the recognition of fibrosing adenosis at the different evolutionary stages and an insistence on paraffin section to confirm all frozen-section diagnoses. This is also accentuated by Haagensen (1956).

Ackerman (1953) summarized his views in an addendum to his remarks on chronic cystic mastopathy. He believes sclerosing adenosis is a highly proliferative form of simple cystic disease of rather uncommon type, with a 1 percent incidence of benign breast lesions, occurring most frequently in the upper-outer quadrant and having a rather disclike configuration. He also mentions the possibile simulation of carcinoma in frozen section but like Mulligan emphasizes lobular configuration as an important diagnostic feature.

Stewart (1950) gives a valuable and detailed consideration of fibrosing adenosis in the section on lesions simulating carcinoma, in his atlas of breast tumors. He suggests that the fibrosing phase may be the one most commonly seen and recognized, but that it is also important to recognize the florid phase of epithelial efflorescence. In his experience the condition is not seen in women under age 20 but may occur after 40, although the majority of cases are found in the third and fourth decades. Stewart also emphasizes that this lesion is especially liable to be overdiagnosed by pathologists, who see only a small number of breast specimens. This is probably a very real danger as in some instances not only mastectomy but postoperative radiotherapy and radiation sterilization were recommended. He especially deprecates the unnecessary "single-breasted" state of not a few young women in the third decade of life because of this diagnostic failure.

Stewart gives detailed accounts of the morbid anatomy of the lesion which is discrete, often coarsely nodular, less firm than carcinoma, and usually distinctly lobulated. Histologically the lesion has a lobulated appearance, regularity of nuclei, and absence of mitoses in the sclerosing phase. In the florid phase, however, mitoses are not infrequent, and some cellular variability may be encountered as well as solid epithelial islands without evidence of lumina. Under high-power magnification, single fields of the florid lesion may be indistinguishable from carcinoma. In these purely florid areas the connective-tissue component scarcely participates. The lesion begins in an intralobular position or in a near-terminal or terminal duct that fails to form normal mammary lobules. In his view the newly formed cell cords never have circumferential myoepithelial investments. Stewart concedes that sclerosing adenosis may occur also in microscopic foci and that the only real difference between this form and the one detected as a palpable mass is that the former occurs in miniature.

As previously mentioned in the discussion on adenosis, Ingleby and Gershon-Cohen (1954) recently reviewed the problem of adenosis generally and restated their views in their monograph (1960). They define adenosis as unencapsulated parenchymatous hyperplasia with single or multiple foci but nearly always bilateral; little has been done in the way of exact anatomic classification. Ingleby and Gershon-Cohen classify adenosis into four types: Type A consists of lobular hyperplasia with very large lobules or a number of smaller lobules close together, while type B, the most common form, consists of lobules with a few more or less dilated ductules of incomplete lobular development. Type C they claim is present near the tumor in most carcinomas of young women and is difficult to distinguish from type B. Epithelial and basal, but not myoepithelial, proliferation may be noted and duct epitheliosis may also be present. The validity of this distinction by type remains uncertain, as I mentioned in my discussion of adenosis in the previous section.

Type D is sclerosing adenosis and Ingleby and Gershon-Cohen think this is often formed entirely of myoepithelial cells. They would therefore prefer to call this lesion "myoid sclerosis" claiming that all stages of myoid development from the large embryonic type of cell, similar to that of early pregnancy, to complete nuclear loss and fibrosis may be seen. When adenomatous nodules undergo involutionary change the epithelium degenerates as in the normal menstrual phase, the myoid nuclei disappear, and only the fibrils are left. These now stain as collagen instead of muscle. The validity of this concept of sclerosing adenosis as well as that of possible precancerous potentiality in their type C adenosis remains uncertain. Urban and Adair (1949) and Foote and Stewart (1945) have published valuable papers on sclerosing adenosis. The latter authors noted that the lesion was present to some degree in 12.5 percent of 200 noncancerous and 7.0 percent of 300 cancerous breasts studied, and these figures do not differ greatly from mine, *i.e.*, 12.2 and 5.3 percent, respectively, in my surgical material.

Dawson (1954) published a very comprehensive and valuable descriptive article on fibrosing adenosis accentuating the fact that it is often unrecognized. She states also the possibility of overdiagnosis and the possibility not only of subjecting the patient to unnecessary operative risk but also of vitiating statistical figures for cancer survival rates, a most important concept. Dawson also re-emphasizes the importance of recognizing a florid, previously proliferative lesion progressing to a fibrotic or sclerotic state. She reviews and clarifies the essentially physiological nature of mammary adenosis and indicates that in itself the term "adenosis" carries no pathological implication. It is seen characteristically in pregnancy, at puberty, and at the menopause and postmenopausal periods. She observes that in older women especially, involution after pregnancy without lactation may be slow, and my studies confirm this observation. Postmenopausal adenosis may, I believe, be due to continued estrogen secretion by the adrenal cortex. Novak (1952) has speculated on a similar basis for the noted occurrence of postmenopausal endometrial hyperplasia seen in women without evidence of granulosa-cell tumor of the ovary. It is of interest to note at this point that Geschickter (1945) illustrates a case of mastodynia following estrogen overdosage. The patient received over 1 million IU of estradiol benzoate over a period of 20 months, and the microphotograph shows distinct sclerosing adenosis.

Dawson points out forcibly that recognition of these phases of normal growth with possible irregularity or perversion is necessary for an understanding of fibrosing adenosis, which is not neoplastic but the regressive stage of a process that begins as an overgrowth of normal or exaggerated physiological pattern.

Dawson found that fibrosing adenosis is most common in younger married women and is frequently associated with some abnormality or perversion of mammary function. However, it does occur in younger, unmarried subjects and in older patients; my findings in surgical material are in accord. She states that fibrosing adenosis as a localized tumor-like condition is rare and my findings also indicate this. It is, however, not uncommon as a focal change in the breast as seen in surgically removed specimens or at autopsy.

In histological studies Dawson, who publishes a large number of photographs, agrees that it is essential to look for the pattern of growth and not concentrate on high-power-view appearances. Dawson's description of the microscopic appearances corresponds closely with those of Mulligan (1951) and Stewart (1950), and my findings correspond closely with all of these. Dawson rightly notes that staining by silver methods or by phosphotungstic acid-hematoxylin shows an intact basement membrane around the distorted glands or cell groups with no evidence of invasion. Mitoses may occur in the epithelial cells in the florid phase and in the fibroblasts in the actively sclerosing phase. She suggests that epitheliosis is absent in the ducts in sclerosing adenosis, but with this my findings are not in full agreement. Although there may be no marked epitheliosis this is by no means invariable, but she concedes that epitheliosis may be seen in association with sclerosing adenosis in older women and that the two are not related. It seems possible to me, however, that disordered sex hormone balance may lead to papillary epitheliosis and papillomatosis, as well as adenosis, and there is no reason why the two should be mutually exclusive. Since it is generally believed that epitheliosis of ducts frequently precedes the development of intraductal and therefore of infiltrative carcinoma, the occasional association of sclerosing adenosis and carcinoma is not surprising. Nevertheless it should be stated that there is no causal relationship between sclerosing adenosis and mammary neoplasia. This view is held by all workers who have studied the lesion, and it is generally accepted that the sclerosing phase represents an involutionary process which becomes practically quiescent.

Results

It may be said at once that sclerosing adenosis, since it is a lobular phenomenon, has not been demonstrated in any of 500 male breasts examined. If we consider first uncomplicated sclerosing adenosis, *i.e.*, sclerosing adenosis, as the sole proliferative change in the breast, the over-all total in the 800 female cases examined was 25, an incidence of 3.1 percent. Of these, 16 occurred in fibrous breasts, *i.e.*, 3.7 percent of all fibrous breasts, and 9 in fatty breasts, 2.4 percent of all fatty breasts. No case was seen under age 36 or over age 80. The quinquennia of maximal incidence were 41 to 45, 51 to 55, 61 to 65, and 71 to 75, the percentages of all cases in these periods being 4.4, 4.1, 5.8, and 5.2. This type of scatter can scarcely be significant unless regarded broadly and is probably a chance effect; over the age group 41 to 75 the incidence is 3.5 percent of all cases.

In these uncomplicated cases there is a virtually even distribution of phases of development of the lesion: 36.0 percent, florid; 36.0 percent, in the intermediate phase; and 28.0 percent, in the sclerosed phase.

The total number of all cases of sclerosing adenosis, whether present as the sole lesion or not, was 56 out of 800 cases, an over-all incidence of 7.0 percent. The maximum quinquennial incidence was in the periods 51 to 55 (10, or 10.2% of all cases), 61 to 65 (14, or 10.1% of all cases), and 71 to 75 (11, or 11.4% of all cases). Of these 56 cases, 37.5 percent were in the florid phase, 35.7 percent in the intermediate phase, and 16.1 percent in the sclerosed phase; the remaining 10.7 percent were distributed evenly between cases showing florid and sclerosed, intermediate and sclerosed, or all three phases. The sclerosed phase in the over-all group of cases was significantly less frequent than the preceding phases. It may be noted here that in 5 cases with florid sclerosing adenosis, apocrine metaplasia was a feature in women aged 59, 62, 64, 71, and 75 (figs. 7 through 12). Acinar calcification was noted in 2 cases, 1 aged 69 in the intermediate phase and 1 aged 50 in the sclerosed phase.

In view of Dawson's assertion that sclerosing adenosis and epitheliosis rarely occur together, it may be mentioned that in 24 of these 56 cases (42.1%) of sclerosing adenosis some degree of epitheliosis or intraductal hyperplasia was present. In 2 there was papillomatosis also and in another occult carcinoma and a fibroadenoma. Of these 24 cases, 15 occurred in fibrous breasts (3.5% of all such breasts) and only 9 in fatty breasts (2.5% of all such breasts); this accords with the total incidence of sclerosing adenosis—of the 56 cases, 37 occurred in fibrous breasts (8.5%) and 19 in fatty breasts (5.2%).

It is interesting that of the 56 cases of sclerosing adenosis only 14 (25%) were bilateral. It is quite probable that if more blocks had been taken this would have materially altered the figures. In my surgical series I demonstrated bilateral florid sclerosing adenosis in a woman who had two mastectomies with an 18-month interval between them.

The figures relating to sclerosing adenosis are found in tables 10, 11, 12, 13, and 14.

Some figures from my studies of 1,010 surgically removed female breasts or portions of breasts are: uncomplicated sclerosing adenosis, 12 cases (1.2%); 6 in the florid phase, the remainder more or less sclerotic. All these women were in the age group 20 to 49, and their condition was such as to require surgical interference. In the majority there was no history of gross menstrual upset.

Sclerosing adenosis, however, as a complicating factor in breast tissue that had been removed for another cause was present in 73 cases (7.2%) giving an over-all incidence for sclerosing adenosis of 85 of 1,010 cases, *i.e.*, 8.4 percent of all cases. Of these 73 complicating cases, 28 were

TABLE 10.—Uncomplicated sclerosing adenosis

Age group	Breast type				Total	Percent of all cases
	Fibrous	Percent of all cases	Fatty	Percent of all cases		
21–25	—		—		—	
26–30	—		—		—	
31–35	—		—		—	
36–40	1	3.7	—		1	3.0
41–45	2	5.9	—		2	4.4
46–50	1	2.6	—		1	1.7
51–55	3	5.6	1	2.3	4	4.1
56–60	—		1	1.7	1	1.1
61–65	4	7.3	4	4.8	8	5.8
66–70	1	2.4	1	1.4	2	1.8
71–75	3	5.9	2	4.3	5	5.2
76–80	1	4.2	—		1	2.7
81–85	—		—		—	
86–90	—		—		—	
21–90	16	3.7	9	2.4	25	3.1

TABLE 11.—Uncomplicated sclerosing adenosis

Age group	Phase of development						Total
	Florid		Intermediate		Sclerosed		
	Number	Percent	Number	Percent	Number	Percent	
21–25	—		—		—		—
26–30	—		—		—		—
31–35	—		—		—		—
36–40	—		1	100	—		1
41–45	—		1	50	1	50	2
46–50	1	100	—		—		1
51–55	1	25	2	50	1	25	4
56–60	—		1	100	—		1
61–65	2	25	3	37.5	3	37.5	8
66–70	1	50	—		1	50	2
71–75	3	60	1	20	1	20	5
76–80	1	100	—		—		1
81–85	—		—		—		—
86–90	—		—		—		—
21–90	9	36.0	9	36.0	7	28.0	25

carcinomatous, 6 papillomatous, 2 fibroadenomatous, 13 showed epitheliosis (hyperplastic cystic disease), 24 showed simple fibrocystic disease, and 1 showed infective mastitis. When the total 85 cases are considered, the maximal incidence occurred in the fifth decade. The incidence by decade was: 37 or 11.5 percent of all cases in the fifth; 15 or 9.6 percent of all cases in the fourth; and 15 or 8.2 percent of all cases in the sixth.

The only paper I have been able to trace that gives figures on the incidence of sclerosing adenosis in surgically removed specimens is that of Foote and Stewart (1945) who noted the lesion in 12.5 percent of 200 noncancerous and 7.0 percent of 300 cancerous breasts. These figures do not greatly differ from mine, *i.e.*, 12.2 for noncancerous and 5.3 percent for cancerous breasts in surgical specimens.

TABLE 12.—All cases of sclerosing adenosis

Age group	Breast type					
	Fibrous		Fatty		Total	
	Number	Percent	Number	Percent	Number	Percent
21–25	—		—		—	
26–30	—		—		—	
31–35	—		—		—	
36–40	2	7. 4	—		2	6. 1
41–45	2	5. 9	—		2	4. 4
46–50	4	10. 5	—		4	6. 9
51–55	9	16. 7	1	2. 3	10	10. 2
56–60	1	2. 9	3	5. 1	4	4. 2
61–65	7	12. 7	7	8. 4	14	10. 1
66–70	2	4. 8	3	4. 1	5	4. 4
71–75	7	13. 7	4	8. 7	11	11. 3
76–80	3	12. 5	—		3	8. 1
81–85	—		1	25. 0	1	8. 3
86–90	—		—		—	
21–90	37	8. 5	19	5. 2	56	7. 0

None of the authors who have published reports of series of postmortem breast specimens seem to have differentiated sclerosing adenosis from their other findings. Indeed there are many pathologists who classify lesions of sclerosing adenosis as forms of hyperplastic disease or epitheliosis, instead of as a variant of the large group of "adenosis" which is undoubtedly the correct category for the lesion. With regard to possible endocrine relationships, I have already pointed out that in cases of uncomplicated sclerosing adenosis in my surgical series there was no history of gross menstrual upset in the majority. One of the women, who had no history of menstrual upset, had undergone an abdominal operation 3 weeks before the mammary biopsy, at which time her pelvic organs had been entirely normal. In 1 woman, aged 24, however, the mass had appeared in the 5th month of her only pregnancy 2 years before operation; the child was not breast fed and for 7 months before operation she had suffered severe menorrhagia. Another woman aged 44 also had menorrhagia for 3 months before operation.

One 49-year-old woman, in which sclerosing adenosis complicated carcinoma, had, shortly before mastectomy, undergone a course of estrin therapy for menopausal symptoms. Another woman aged 64 died several days after mastectomy and at autopsy had a rather hyperplastic endometrium, with florid sclerosing adenosis in both the remaining breast and in the surgically removed one.

In my autopsy series proper I have examined the ovaries in 16 cases of sclerosing adenosis—in 11, the ovarian stroma was +++ cellular; in 4, ++ cellular; and in 1, + cellular. However, it must be noted that a considerable proportion of women (47.6%) with ovarian stroma +++ cellular showed atrophic breast parenchyma, and this finding is probably not significant. In several cases, the endometrium also varied from a hyperplastic to an inactive appearance. In 1 of these having a hyper-

Table 13.—All cases of sclerosing adenosis*

Age group	Phase of development												Total
	Florid		Intermediate		Sclerosed		Florid/sclerosed		Intermediate/ sclerosed		All phases		
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	
21–25	—		—		—		—		—		—		—
26–30	—		—		—		—		—		—		—
31–35	—		—		—		—		—		—		—
36–40	1	50	1	50	—		—		—		—		2
41–45	—		1	50	1	50	—		—		—		2
46–50	2	50	1	25	1	25	—		—		—		4
51–55	2	20	4	40	2	20	1	10	—		1	10	10
56–60	2	50	1	20	—		—		1	20	—		4
61–65	5	35. 7	4	28. 6	3	21. 4	1	7. 1	1	7. 1	—		14
66–70	3	60. 0	1	20. 0	1	20. 0	—		—		—		5
71–75	4	36. 4	5	45. 5	1	9. 05	—		—		1	9. 05	11
76–80	1	33. 3	2	66. 7	—		—		—		—		3
81–85	1	100. 0	—		—		—		—		—		1
86–90	—		—		—		—		—		—		—
21–90	21	37. 5	20	35. 7	9	16. 1	2	3. 5	2	3. 5	2	3. 5	56

*Acinar calcification noted in 2 cases, 1 intermediate phase, aged 69; 1 sclerosing, aged 50. Apocrine metaplasia noted in 5 florid cases, aged 59, 62, 64, 71, and 75.

Table 14.—Sclerosing adenosis with epitheliosis

Age group	Breast types		Total	
	Fibrous	Fatty	Number of cases	Percent of all cases
21–25	—	—	—	
26–30	—	—	—	
31–35	—	—	—	
36–40	1	—	1	3. 0
41–45	—	—	—	
46–50	1 (occult carcinoma, fibroadenoma)	—	1	1. 7
51–55	3 (1 adenosis also)	—	3	3. 1
56–60	1	2	3	3. 2
61–65	3	3 (1 papillomatosis also)	6	4. 4
66=70	1	1	2	1. 8
71–75	3 (1 papillomatosis also)	2	5	5. 2
76–80	2	—	2	5. 4
81–85	—	1	1	8. 3
86–90	—	—	—	—
21–90	15 (3.5%)	9 (2.5%)	24	3. 0

plastic endometrium, there was a granulosa-cell tumor of one ovary. Another woman who showed sclerosing adenosis had had both ovaries removed some time previously.

The appearance of the pituitary varied and in some cases appeared entirely normal; in others there was slight basophil hyperplasia; 2 showed a chromophobe adenoma and another a tiny beta-cell adenoma; 1 showed an alpha-cell adenoma with thyroprival change in a case of myxedema; and another woman had had a portion of her pituitary destroyed by radon about 2 weeks before death. In fact there was no morbid histological finding in ovary or pituitary which occurred constantly in sclerosing adenosis.

Summary

In postmortem specimens of breast, uncomplicated sclerosing adenosis occurred in 3.1 percent of all cases examined. It was seen only between the ages of 36 and 80 and occurred more frequently in fibrous than in fatty breasts, and also more frequently in the florid or intermediate phase than in the sclerosed. In contrast, in surgically removed breast specimens, uncomplicated sclerosing adenosis was seen in only 1.2 percent of 1,010 specimens, all occurring in the third or fourth decade. These latter cases, however, were such that the condition had led to surgical interference, which places them in a totally different category.

In cases of sclerosing adenosis epitheliosis was the most frequent complicating lesion in the postmortem series, while in the surgical series carcinoma, papilloma, and epitheliosis were all primary causes for the tissue resection.

If all cases showing sclerosing adenosis are considered together in the postmortem series, the over-all incidence was 7.0 percent, and in the surgical series 8.4 percent. In the postmortem series the maximal incidence was in the age group 51 to 75 and in the surgical series 30 to 59.

In the postmortem series the sclerosed phase of sclerosing adenosis was less frequent than the preceding phases.

There was no constant finding in the ovary or pituitary which occurred constantly in, or is peculiar to, sclerosing adenosis.

FIBROADENOMA

Fibroadenoma is one of the more common neoplasms of the breast. In my surgical series of 1,010 female cases (1958) I found 110 fibroadenomas. Of these, 9 occurred in teen-age girls and 8 in women over age 50. The remainder constituted 51.4 percent of all cases in the decade 20 to 29 years, 21.6 percent in the decade 30 to 39, and 5.6 percent in the decade 40 to 49. Only 6 of these fibroadenomas were of the giant variety and the majority occurred in the decade 60 to 69. I have shown, however, in a previous study (1955) that giant fibroadenomas occur in women in the decade 30 to 39, and that "fetal" fibroadenoma occurs in teen-age girls.

For reviews of our knowledge of fibroadenoma, reference may be made to Cheatle and Cutler (1931) and to Willis (1960). An excellent monograph describing 77 cases of cystosarcoma phyllodes is available from the Memorial Hospital (Treves and Sunderland 1951). A further study by Lester and Stout (1954) is of considerable value. These authors give an adequate review of the condition, and reference to Brodie's (1846) original paper "On Serocystic Tumour of the Female Breast" is of considerable interest. For an account of adenomas of the breast, and a report of carcinomatous changes, reference may be made to Hill and Miller (1954) and to Smith (1949). Dawson (1960) discusses in detail breast sarcomas and their relation to fibroadenomas.

Frantz *et al.* (1951) give the only postmortem figures of which I am aware. Of their 225 cases, no less than 21 showed fibroadenoma. The ages ranged from the teen-age group to the decade 80 to 89. However, only 4 were gross and 17 microscopic. All 4 gross cases were more than 40 years of age and in 2 no other lesion was found. Of the others, 1 had gross cysts and 1 had hyperplastic cystic disease.

In the present series only 4 fibroadenomas were noted—an incidence of 0.5 percent. In several cases, however, small fibroadenomatous areas were noted, which in my opinion did not qualify as neoplasms. I feel that Frantz *et al.* classified some such areas as microscopic fibroadenomas. In the 4 cases, the ages were 35, 54, 54, and 74 years; 3 were accompanied by adenosis and the 4th was present in an unexceptional atrophic breast. All were about 1.0 cm in diameter and 3 were hyalinized. Two were intracanalicular; 1, pericanalicular; and the 4th, of mixed appearance (fig. 13).

Summary

True fibroadenoma is uncommon in postmortem breasts. In this series the over-all incidence was 0.5 percent. The majority was hyalinized and showed adenosis of the surrounding breast tissue.

EPITHELIOSIS AND PAPILLOMATOSIS

These epithelial proliferative lesions are considered together because of the undoubted transition that is sometimes seen from papillary epithelial hyperplasia through papillomatosis to acceptable papilloma with well-formed connective-tissue cores bearing blood vessels. In this view I am supported by Willis (1960).

I have chosen to use the term "epitheliosis" defined by Dawson (1948) as ". . . the multiplication of epithelial cells within existing gland structures without formation of new gland elements . . . a cellular filling of the lobule, not a branching, which indicates a more or less pathological response, though it may be only a temporary activity and be followed by cell degeneration and cyst formation." This definition clearly separates epitheliosis from adenosis, which is an essentially branching process to form new gland elements. In the involuting breast, as we have seen, there may be an adenosis which can regress in time. However, if the adenosis is sufficient to be clinically obvious there is usually some degree of epitheliosis, with or without consequent cyst formation that follows degenerative change. While adenosis never proceeds to neoplasia, this appears to be definitely not the case with epitheliosis.

It is here that we encounter great difficulties because of the wide variety of terms used to describe the process of epitheliosis, which may be accompanied by cyst formation and adenosis. It is my opinion that epitheliosis is the key pathological change in the involuting breast, and for that reason I have considered it without reference to secondary changes which seem to me unimportant. In the older literature great stress was laid on the cystic element of the process; this is not surprising because macrocyst formation is clinically obvious and it is impossible to decide clinically whether epitheliosis is present in a breast. The term commonly used by the clinician is still "chronic mastitis," and Boyd (1945) in his textbook of surgical pathology reluctantly retains this term only because of its sanction by long-continued and almost hallowed usage. Illingworth and Dick (1956) in their textbook of surgical pathology also retain the term "chronic mastitis" but equate it with "fibroadenosis." They do, however, introduce the terminology of "adenosis" and "epitheliosis" in their discussion of the significance of "chronic mastitis." Beattie and Dickson (1943) in their textbook, based on the Edinburgh School, use the name "fibroadenosis" and it seems reasonable to assume that Illingworth and Dick, who are practicing surgeons, have been influenced by the pathological terminology of their former school.

Ewing (1940) favors the qualification of "chronic productive mastitis" to accentuate the proliferative nature of the process, while Geschickter (1945) prefers the term "chronic cystic mastitis" and Haagensen (1956) "cystic disease of the breast." Kuzma (1953), in Anderson's textbook of pathology, prefers the name "mammary dysplasia" which seems non-committal and very comprehensive—reminiscent of such terms as "dermatosis" or "lymphadenopathy." Willis (1960) adopts the phrase "cystic hyperplasia." Perhaps the most accurate and descriptive term is that used by Muir (1941) in his textbook, and later adopted by Cappell (1958) in the most recent edition of this standard work: "Hyperplastic cystic disease" which at once describes two of the essential phenomena, hyperplasia and cyst formation.

Other terms still sometimes heard are "Schimmelbusch's disease" and "Reclus' disease" but these are now largely of historic interest only. The older authors were largely obsessed by the importance of the cystic component and elaborate classifications were made based on the frequency, size, and appearance of the cysts. It may be of interest to note that Schultz (1933) in the most recent edition of Henke and Lubarsch's Encyclopaedia discussed involutional changes under the generic term of "die Zystenmamma." An excellent review of the older literature is given by Semb (1928) in his monograph on "Fibroadenomatosis Cystica Mammae." The work of Bloodgood (1921) ought also to be mentioned.

For teaching purposes, I tend to use the phrase "cystic mastopathy" as a comprehensive term for the appearance of naked-eye cysts in a fibrous breast parenchyma. If histological examination reveals no epitheliosis then the term "simple cystic disease" or "fibrocystic disease" may be applied. I do not consider simple adenosis or sclerosing adenosis out of place in simple cystic disease. If, however, epitheliosis is present, then Muir's phrase "hyperplastic cystic disease" may be employed, although the term "epitheliosis" itself may be used since we believe that cystic change in these cases is not an important feature, when compared with the at least potentially dangerous hyperplastic component. Frequently cyst formation is not marked despite considerable epitheliosis, and for this reason a pathologically significant lesion may not be clinically detected. Conversely, a cystic breast which excites surgical interest may show no evidence of epitheliosis and may be dismissed by the pathologist as of no great interest.

Perhaps this somewhat explains the divergence of opinion between many pathologists and surgeons on the significance of cystic disease as a possible precursor of carcinoma. Often such protagonists have not defined their premises and thus argue at cross purposes about different entities. The surgeon is interested in detectable simple cystic disease and the pathologist in hyperplastic cystic disease, in which the cysts may be microscopic, and no common meeting ground is discovered.

In the development of modern thought on mammary epitheliosis, *i.e.*, epithelial hyperplasia, the important work of Cheatle must not be overlooked. In his paper of 1926 he clearly states that, in his opinion, there is a definite sequence of events in epithelial change in the breast that ends in carcinoma. He does not claim that these changes can invariably be detected in all breast cancers. He places the significant site for this epithelial change in the terminal ducts and the acini and notes that two phases occur, a so-called "cystiphorous desquamative epithelial hyperplasia" which may progress to a more important stage of dysgenetic epithelial hyperplasia that may be papillary or become an actual intraductal precancerous condition which later becomes invasive. These views were later elaborated with a wealth of evidence by Cheatle and Cutler (1931) in their great work on tumors of the breast, which was based on a painstaking study of many large sections. Although the terms "desquamative" and "dysgenetic" are now rarely used, the essential concept of epithelial hyperplasia is accepted and it is this phenomenon which we mean by the term "epitheliosis."

The papers of Muir (1934, 1935, 1939, 1941) on the evolution of carcinoma of the mamma also emphasized the importance of intraductal and intra-acinar epithelial hyperplasia as a possible precursor of breast neoplasia. Muir's 1941 paper is fully and beautifully illustrated and presents a series of stages in this process which can readily be appreciated. Muir does not claim that this evolutionary sequence can always, or indeed frequently, be traced: Proliferative changes in the human breast may be focal, possibly due to a varying reactivity of different parts of the epithelial system to the presumptive hormonal stimulus. It is interesting that the response of the mouse breast to estrogen stimulation may not necessarily be uniform but is sometimes variable and often focal also. Carcinoma of the breast may sometimes be of multicentric origin, but confusion may arise from intraepithelial spread of malignant cells along the duct systems. Muir not only carefully detailed a developmental sequence of neoplasia from preceding epitheliosis, which may be papillary or cribriform in type and sited in the ducts, but also noted acinar epithelial hyperplasia which may be difficult to separate sharply from actual intra-acinar carcinoma. Muir rightly points out also the characteristic cribriform pattern of many intraductal carcinomas and here again the points of distinction between marked hyperplasia and early neoplasia may be very fine.

The work of Dawson corroborates many of Muir's findings and her lecture (1948) to the Royal College of Surgeons of England fittingly details her views on the genesis of carcinoma. Most pathologists agree that, in at least some cases, the evolutionary progress from epitheliosis through intraductal carcinoma to infiltrative tumor can be traced. Willis, indeed, has said (1960) "Any competent pathologist who has examined a considerable number of surgically removed breasts will find it difficult to remain patient with those surgeons who deny the precancerous potentialities of cystic hyperplasia."

On the other hand Willis admits ". . . it is not denied that some breast cancers are of localized origin and unaccompanied by significant changes in other parts of the organ." But he also states ". . . the more thoroughly the pathologist examines amputated breasts, the more often will he discover the presence of cystic hyperplasia in association with carcinoma and of clear transitions from one to the other."

However, some authorities discount histological appearances and the alleged evolutionary sequences as being only the formation of a sort of selective jigsaw puzzle where pieces are taken or discarded only to suit the convenience of the proponents of the evolutionary schemes. In this connection Foulds' (1958) statement may be borne in mind, "In the light of experimental studies some disputed precancerous lesions are inessential collateral manifestations of a stimulus that is essential to neoplasia. Chronic mastitis, for example, is perhaps more important as an indicator of excessive estrogen stimulation than as an immediate precursor of mammary cancer." There is a school in the United States which believes that cancer may develop in any type of breast parenchyma; this view is summed up in Stewart's (1950) dictum that "the female breast is a precancerous organ." Stewart's views are largely based on the result of a study with Foote (Foote and Stewart 1945) of "cancerous versus noncancerous breast." This study involved a meticulous examination of 300 radical mastectomies for carcinoma, 200 mastectomies or adequate biopsies for nonmalignant conditions, and 27 postmortem specimens obtained from the medical examiner (corner's pathologist). The authors make the provocative statement ". . . the term 'chronic cystic mastitis' is so ingrained in the minds of some pathologists that this diagnosis of a locally excised portion of breast almost amounts to a surgico-pathologic reflex."

Foote and Stewart claim that they have traced the origin of mammary carcinoma from duct papilloma, solitary and multiple cysts, "apocrine" epithelium, and from blunt duct adenosis. They suggest that some women possess mammary epithelium which is more than normally responsive to estrogen stimulation in the same way that other women seem to have a more responsive endometrium. This detailed work perhaps deserves to be fully summarized and I am therefore giving what I understand to be their most important conclusions: Macroscopic cysts are more common in noncancerous than cancerous breasts; in cancerous breasts coexistent papillomas show cytological atypia 5 times more frequently than in noncancerous breasts and cancer may develop from such atypical papillomas; and duct stasis, duct ectasia, and periductal mastitis are not related to developing carcinoma. The latter opinion contrasts with the views of Keynes (1923–24), Cappell (1931), and Willis (1960) that retained duct secretions may play a part in carcinogenesis. Foote and Stewart further state that intralobular hyperplasia (intra-acinar epitheliosis) may merge into acinar carcinoma, a point on which there is considerable agreement.

Among other points brought out by these workers is one that may be overlooked because it is obvious, namely, that the frequency of "chronic cystic mastitis" depends on what lesions, and how many, are necessary to establish the diagnosis. Foote and Stewart include in this category: cysts, duct papillomatosis, blunt duct adenosis, apocrine epithelium, and sclerosing adenosis. They concede that morphological and statistical studies indicate that cystic mastitis plays a part in the development of breast carcinoma, though they decline to give an opinion on the extent. They believe that the important factor is the papillary epithelial hyperplasia of atypical type, which in some persons appears to escape from the normal restraint exercised on the body tissues.

Warren (1940) demonstrated that patients with "chronic mastitis" showed a distinct predisposition to mammary carcinoma, *i.e.*, 4.5 times more frequent than in the general population. In contrast Geschickter (1945) believes that " . . . histologic study made on human breast tissue cannot be cited as convincing evidence of the transition of hyperplasia or involutional dysplasia into malignancy so long as morphologic criteria are lacking to distinguish between benign neoplasia or early malignancy." Geschickter seems to believe that an added stimulus is required to produce the malignant change in mammary dysplasia. Rodman (1935) and Logie (1942) think that there is a relation between "chronic mastitis" and carcinoma. Haagensen (1956) clearly destinguishes between epithelial proliferation and intraductal carcinoma and apparently does not regard the former as being serious.

The foregoing review of opinions indicates something of the opposing views on this vexing question. I believe progress might be made toward its solution if there were general agreement on a suitable term to define the essential lesion of epithelial hyperplasia, and I am of the opinion that Dawson's "epitheliosis" is an entirely suitable one.

I turn now to postmortem studies of which no great number exist: Keynes (1923–24) examined 116 postmortem specimens of breast and bacteriological and histological examinations revealed no significant evidence of a microbiological cause for "chronic mastitis." Of these 116, he decided that 57 were normal and 59 showed chronic mastitis. His data are not given in sufficient detail to estimate the frequency of epitheliosis in his cases. His criteria for classification as chronic mastitis included epithelial changes. However, it is inconceivable that epitheliosis should have occurred in such a high proportion of cases.

Semb (1928) examined 64 breasts from 32 patients at autopsy: Of these, 6 patients (12 breasts) were either in the pregnant or lactating state, and in the remaining 26 he found microscopic epithelial proliferation in only 1, an incidence of less than 4 percent. Charteris (1930) found 5 cases of chronic mastitis in 32 breasts examined at autopsy, an incidence of 15.6 percent. Lindgred (1936–37) studied 120 breasts obtained at autopsy from women whose ages ranged from 35 to 75 and found "cystiphorous epithelial hyperplasia" in 16 glands, an incidence of 13.3 percent.

Borchardt and Jaffe (1932) examined the breasts of 100 women over the age of 40 at autopsy. They found mammary dysplasia or atypical involution in 93 percent of all cases (in 70% this was bilateral). Franzas (1936) also examined the breasts of 100 women between the ages of 18 and 98 years and found "chronic mastitis" in 55 percent (in 25% this was bilateral). Walchshofer (1930) in postmortem examinations of 65 women between the ages of 19 and 84 also found hyperplastic changes, which he illustrates in very indifferent photographs. Geschickter (1945) examined the breasts of 100 women, aged between 30 and 50 years, at autopsy and found that in the age group 30 to 40, 33 percent showed lobular irregularities and cystic dilatation, while in the age group 40 to 50 the figure was 55 percent. In all these instances the findings are not detailed sufficiently to elucidate firm figures of the incidence of epitheliosis.

Berning and Bücker (1937), however, give some details of the breast structure at autopsy in 50 women whose ages range from 16 to 60 years and it appears that some degree of papillary hyperplasia was present in 20 to 25 percent. In the largest study, which I have traced, Frantz *et al.* (1951) examined the breasts of 225 women at autopsy, the ages ranging from 13 to 88 years, and found intraductal proliferation in 36, an incidence of 16 percent. Of these, 7 were associated with adenosis, 19 with cyst formation, and 31 with apocrine metaplasia. The age range and calculated incidence per decade are shown:

Age group		Percent
20–29	1	(7.1)
30–39	3	(10.0)
40–49	9	(20.9)
50–59	8	(14.0)
60–69	7	(15.9)
70–79	4	(19.0)
80–89	4	(44.4)

Of these 36 cases, 21 showed a papillary pattern, *i.e.*, 58.3 percent, while the remainder were of solid type. Of the 36, only 7 were presumed to be premenopausal.

Results

If we first consider epitheliosis as a pure lesion unaccompanied by any other proliferative change, we find that 103 of 800 cases fall into this group, an over-all incidence of 12.9 percent. These occurred only in the period 41 to 85 years and if we examine the incidence in this period alone we find that uncomplicated epitheliosis occurred in 103 of 693 cases, *i.e.*, in 14.9 percent.

If we next consider the occurrence of pure epitheliosis in relation to the breast parenchyma, we find that of these 103 cases 36 occurred in 434 fibrous breasts, 8.3 percent, and 67 in 366 fatty breasts, 18.3 percent. As we shall see later this is paralleled, when we consider all cases of epitheliosis. Predominance of pure epitheliosis in fatty breasts begins at the period 56 to 60 so that it would appear that women older than this, whose breast atrophy is not accompanied by fatty infiltration or is not the result of it, are less likely to develop epithelial hyperplasia.

Pure epitheliosis was not seen in women under age 41, although cases accompanied by other lesions, most frequently adenosis, were occasionally seen. This suggests that whatever the cause of intraductal hyperplasia or epitheliosis may be, it does not manifest itself in the younger woman without adenosis or lobular hyperplasia. A hormonal disturbance would seem to be the most likely explanation.

Epitheliosis, whether pure or accompanied by other proliferative lesions, occurred in 176 of 800 cases, an incidence of 22.0 percent, *i.e.*, more than

1 woman in 5 between the ages of 21 and 90 at autopsy, in this department, showed some degree of epitheliosis. The age incidence spreads from 26 to 85, though the condition only becomes frequent after age 41. There is a sharp rise in incidence from 6.1 percent in the quinquennium 36 to 40 to 26.7 percent in the quinquennium 41 to 45. This is interesting in view of Dawson's (1943) findings, which I have confirmed in my studies of surgical material, that under age 40 the majority of breast lesions are benign and that over age 50, the majority are malignant. Dawson's figures were 85 and 85 percent, respectively, and my findings in a survey of 531 cases received in the Pathology Department, Western Infirmary, Glasgow, in the years 1948–49 were 84 and 82 percent, respectively—a remarkably close correlation.

Once again we find that overall the condition predominated in fatty breasts, 27.6 as against 17.3 percent in fibrous breasts. This predominance begins at the quinquennium 56 to 60 as in women showing uncomplicated epitheliosis. The severity of change in the majority of cases (68.2%) is mild, while 25 percent is moderately severe, and only 6.8 percent marked. Of the marked cases 3 or 25 percent occurred before the menopausal age and the remainder after the upper limit for this. Of the moderately severe cases the majority (79.6%) occurred after age 50. This distribution is also true of the milder cases; 88.3 percent occurred after age 50.

Overall, the site of epitheliosis is most often confined to the ducts (73.4%), with 20.4 percent affecting both ducts and acini and only 6.2 percent affecting the acini (or ductules as they are termed by some workers). It is noteworthy that in women under age 40 the acini are never affected and that under age 50 the acini are never affected alone. In the present series, affection of the acini alone was seen in only the restricted age period from 51 to 70, although affection of ducts and acini together was seen up to age 80.

Turning now to patterns of epitheliosis or epithelial hyperplasia we find that the commonest form is the papillary type which in its pure form accounts for 47.1 percent of all cases, but if considered in association with other patterns totals 66.5 percent. Cribriform and solid patterns are much less common and account in their pure forms for only 15.9 and 13.1 percent of cases, respectively (figs. 14 through 21).

Of the 12 severe cases, 6 were unaccompanied by other lesions but 3 were accompanied by carcinoma and 1 by papillomatosis. Of the 44 moderately severe cases 23 were unaccompanied by other lesions, 6 were accompanied by papillomatosis, 3 by occult carcinoma, and 3 by clinical carcinoma. Of the 120 mild cases, 78 were unaccompanied by other lesions but 6 were accompanied by papillomatosis, 2 by clinical carcinoma, and 1 by occult carcinoma. *See* tables 15 through 20.

Turning now to the question of papillomatosis it may be repeated that the distinction between marked papillary epitheliosis and papillomatosis is a vague one as Willis (1952) has reiterated and Cheatle and Cutler (1931) originally noted. With this view I am in complete agreement,

Table 15.—Pure epitheliosis*

	Breast type					
Age group	Fibrous	Percent of all cases	Fatty	Percent of all cases	Total percent of all cases	
21–25	—	—	—	—	—	—
26–30	—	—	—	—	—	—
31–35	—	—	—	—	—	—
36–40	—	—	—	—	—	—
41–45	5	14. 7	1	9. 1	6	13. 3
46–50	1	2. 6	2	10. 0	3	5. 2
51–55	6	11. 1	3	6. 8	9	9. 2
56–60	2	5. 7	10	16. 9	12	12. 8
61–65	9	16. 4	16	19. 3	25	18. 1
66–70	6	14. 6	17	23. 3	23	20. 2
71–75	5	9. 8	11	23. 9	16	16. 5
76–80	2	8. 3	5	38. 5	7	18. 9
81–85	—	—	2	50. 0	2	16. 7
86–90	—	—	—	—	—	—
21–90	36	8. 3	67	18. 3	103	12. 9

*Other complicating conditions: adenosis in fibrous breasts at ages 47 and 54; fibroadenoma in fibrous breast at age 50; papillomatosis in fibrous breast at age 55; occult intraductal carcinoma and infiltrative carcinoma of breast in fibrous breasts at ages 73 and 68, respectively. Secondary bronchial carcinoma in fibrous breast at age 51.

and I have frequently found it difficult to decide whether to classify a lesion as papillary epitheliosis or as papillomatosis. It is, however, easy to recognize the highly organized solitary or multiple papillomas of larger ducts.

In the series of 800 cases, 10 of papillomatosis were noted and 13 of true papilloma, 1.25 and 1.6 percent, respectively, with an over-all total of 23 cases or 2.9 percent, spread over the age group 36 to 85. Of the true papillomas, 4 were uniradiculate in type and 9 were multiradiculate. Of these, 10 were solitary and only 3 were multiple (figs. 22, 23, and 24). It should be pointed out that if further blocks had been examined this ratio might have been considerably altered. When all cases of papilloma-

Table 16.—All cases showing epitheliosis

	Breast type					
Age group	Fibrous	Percent of all cases	Fatty	Percent of all cases	Total percent of all cases	
21–25	—	—	—	—	—	—
26–30	1	9. 1	—	—	1	8. 3
31–35	2	5. 1	1	20. 0	3	6. 8
36–40	2	7. 4	—	—	2	6. 1
41–45	10	29. 4	2	18. 2	12	26. 7
46–50	4	10. 5	4	20. 0	8	13. 8
51–55	12	22. 2	5	11. 4	17	17. 3
56–60	6	17. 1	16	27. 1	22	23. 4
61–65	13	23. 6	24	28. 9	37	26. 8
66–70	8	19. 5	23	31. 5	31	27. 2
71–75	10	19. 6	16	34. 8	26	26. 8
76–80	6	25. 0	7	53. 8	13	35. 1
81–85	1	12. 5	3	75. 0	4	33. 3
86–90	—	—	—	—	—	—
21–90	75	17. 3	101	27. 6	176	22. 0

Table 17.—All cases of epitheliosis

Age group	Degree						
	Mild		Moderate		Marked		
	Number	Percent	Number	Percent	Number	Percent	Total
21–25	—		—		—		—
26–30	1	100	—		—		1
31–35	2	66. 7	1	33. 3	—		3
36–40	—	—	1	—	1	50	2
41–45	5	41. 6	5	41. 6	2	16. 8	12
46–50	6	75. 0	2	25. 0	—		8
51–55	13	76. 5	4	23. 5	—		17
56–60	16	72. 7	5	22. 7	1	4. 6	22
61–65	27	73. 0	6	16. 2	4	10. 1	37
66–70	23	74. 2	7	22. 6	1	3. 2	31
71–75	17	65. 4	7	26. 9	2	7. 7	26
76–80	6	46. 1	6	46. 1	1	7. 8	13
81–85	4	100. 0	—		—		4
86–90	—		—		—		—
21–90	120	68. 2	44	25. 0	12	6. 8	176

tosis or papilloma are considered together, 13 were associated with epitheliosis (6 of these being papillomatosis and 7 true papillomas), 4 with adenosis, and 3 with sclerosing adenosis. Of the 10 cases of papillomatosis, 3 occurred in fibrous and 7 in fatty breasts, while with true papilloma 6 occurred in fibrous and 7 in fatty breasts (tables 21 and 22).

It may be of some interest to compare these figures with those obtained in my surgical series (1958). Papilloma of ducts was seen in 37 of 1,010 cases, just under 3.7 percent, while epitheliosis was seen as the lesion leading to surgery in 98 cases, just under 9.8 percent of cases. Duct papilloma was twice noted in teen-age girls, but was most common in the fifth decade. Epitheliosis or hyperplastic cystic disease was also most common in the fifth decade and only about half as common in the fourth. Ex-

Table 18.—All cases of epitheliosis

Age group	Patterns of epitheliosis*							
	Papillary	Cribriform	Solid	P/C	P/S	C/S	C/S/P	Total
21–25	—	—	—	—	—	—	—	—
26–30	1	—	—	—	—	—	—	1
31–35	—	1	1	1	—	—	—	3
36–40	—	—	2	—	—	—	—	2
41–45	8	—	2	—	2	—	—	12
46–50	4	—	2	1	—	1	—	8
51–55	9	2	1	2	2	1	—	17
56–60	13	2	5	2	—	—	—	22
61–65	20	3	5	7	—	2	—	37
66–70	11	11	2	4	2	1	—	31
71–75	10	7	2	3	1	2	1	26
76–80	5	—	1	2	3	1	1	13
81–85	2	2	—	—	—	—	—	4
86–90	—	—	—	—	—	—	—	4
21–90	83 (47.1%)	28 (15.9%)	23 (13.1%)	22	10	8	2	176

*P/C = papillary/cribriform; C/S = cribriform/solid; P/S = papillary/solid; C/S/P = cribriform/solid/papillary.

TABLE 19.—All cases of epitheliosis

Age group	Site of epitheliosis: Ducts Number	Ducts Percent	Acini Number	Acini Percent	Ducts and acini Number	Ducts and acini Percent	Totals
21–25	—		—		—		—
26–30	1	100. 0	—		—		1
31–35	3	100. 0	—		—		3
36–40	2	100. 0	—		—		2
41–45	7	58. 3	—		5	41. 7	12
46–50	6	75. 0	—		2	25. 0	8
51–55	9	43. 0	4	23. 5	4	23. 5	17
56–60	15	68. 2	3	13. 7	4	18. 1	22
61–65	29	78. 4	2	5. 4	6	16. 2	37
66–70	24	77. 5	2	6. 4	5	16. 1	31
71–75	21	80. 8	—		5	19. 2	26
76–80	8	61. 5	—		5	38. 5	13
81–85	4	100. 0	—		—		4
86–90	—		—		—		—
21–90	129	73. 4	11	6. 2	36	20. 4	176

TABLE 20.—All cases of epitheliosis*

Breasts	Mild Number	Mild Percent	Moderate Number	Moderate Percent	Marked Number	Marked Percent	Total Number	Total Percent
Fibrous	54	72. 0	18	24. 0	3	4	75	17. 3
Fatty	66	65. 4	26	25. 7	9	8. 9	101	27. 6
	120	68. 2	44	25. 0	12	6. 8	176	22. 0

*Of all women with fibrous breasts 17.3 percent showed epitheliosis, only 28 percent moderate or marked; 27.6 percent of all women with fatty breasts showed epitheliosis, 34.6 percent moderate or marked; 22.0 percent of all women examined showed epitheliosis, 31.8 percent moderate or marked.

TABLE 21.—Papilloma and papillomatosis*

Age group	Papillomatosis†	Papilloma‡	Total Number	Total Percent
21–25	—	—	—	
26–30	—	—	—	
31–35	—	—	—	
36–40	—	1	1	3. 0
41–45	1	—	1	2. 2
46–50	1	—	1	1. 7
51–55	1	3	4	4. 1
56–60	1	—	1	1. 1
61–65	2	2	4	2. 8
66–70	3	1	4	3. 5
71–75	1	3	4	4. 1
76–80	—	2	2	5. 4
81–85	—	1	1	8. 3
86–90	—	—	—	
21–90	10 (1. 25%)	13 (1. 6%)	23	2. 9

*Associated with epitheliosis in 13 cases; with adenosis in 4; with sclerosing adenosis in 3; and sole lesion in 4.

†Papillomatosis: 1 hypovirginal; 2 atrophic; and 7 adipose/obese breasts.

‡Papilloma: 1 virginal; 5 atrophic; and 7 adipose/obese breasts.

Table 22.—Papillomatosis

Postmortem No.	Age	Breast type	Site	Type	Associated with
7523	75	Hyper-virginal	Duct	Papillomatosis	Moderate papillary cribriform epitheliosis: sclerosing adenosis intermediate
7599	59	Adipose	Duct	Papillomatosis	—
7742	55	Atrophic	Duct	Multiradiculate papillomas	Diffuse sclerosing adenosis, all phases
7796	69	Adipose	Duct	Small multiradiculate papilloma	—
7861	75	Adipose	Cyst	Uniradiculate papilloma (apocrine metaplasia)	—
7916	72	Adipose	Cyst	Uniradiculate papilloma (hyalinizing)	Marked solid, cribriform papillary epitheliosis: sclerosing adenosis intermediate
7937	47	Adipose	Duct	Papillomatosis	Moderate solid epitheliosis
8126	73	Adipose	Duct	Multiradiculate papillomas	Moderate papillary epitheliosis in ducts and acini
8146	78	Adipose	Duct	Multiradiculate papilloma	Mild papillary epitheliosis
8262	51	Atrophic	Duct	Multiradiculate papilloma (hyalinizing)	Mild papillary and cribriform epitheliosis
8321	67	Atrophic	Duct	Papillomatosis	Moderate papillary epitheliosis
8345	61	Obese	Duct	Papillomatosis	Mild papillary epitheliosis: adenosis
8347	76	Atrophic	Duct	Multiradiculate papillomas	Moderate papillary epitheliosis
8584	69	Adipose	Duct	Papillomatosis	Mild papillary epitheliosis
8791	40	Virginal	Duct	Multiradiculate papilloma	Adenosis
8921	63	Obese	Duct	Uniradiculate papilloma	—
9001	53	Atrophic	Duct	Uniradiculate papilloma	Adenosis
9068	52	Obese	Duct	Papillomatosis	Adenosis
A9631	82	Atrophic	Duct	Multiradiculate papilloma	Mild cribriform epitheliosis: adenosis
9713	63	Adipose	Duct	Papillomatosis	Mild papillary epitheliosis
9812	42	Atrophic	Duct	Papillomatosis	Moderate papillary epitheliosis: adenosis
9820	64	Obese	Cyst	Small multiradiculate papilloma	Adenosis
B0041	68	Adipose	Duct	Papillomatosis	—

pressed, however, as percentages of all cases in those periods the figures are close, 16.7 and 15.3 percent, respectively.

In the 51 cases showing epitheliosis or papillomatosis in which the ovaries were examined, 28 showed a highly cellular stroma, 12 a moderately cellular stroma, and only 3 a poorly cellular stroma. Of these cases, 2 showed granulosa-cell tumor, and 1 secondary carcinoma; in 5 cases oophorectomy had previously been carried out for reasons other than treatment of breast carcinoma. Some 18 of the ovaries showed microcysts, 4 follicular or luteal activity, and 3 prominent hilus interstitial cells.

The pituitaries from these 51 cases showed no constant appearance either. In 9 the beta cells appeared hyperplastic, in 4 the chromophobe cells, in 3 the alpha cells, and in 1 both the alpha and beta cells. Chromophobe adenoma was noted in 5 and beta-cell adenoma in 1; in 2 there were Rathke-type cysts and in 7 fetal-type chromophobes were evident. In 6 cases the stroma was prominent and hyalinized. In neither ovary nor pituitary was any constant significant feature noted.

Summary

The important factor in "hyperplastic cystic disease," "chronic mastitis," "cystic hyperplasia," "mammary dysplasia," "fibroadenosis," or "involutional dysplasia" is the presence of epithelial hyperplasia or epitheliosis. There is a gradation from papillary epithelial hyperplasia through papillomatosis to papilloma. Although there is considerable debate on the question, the general consensus is that epithelial hyperplasia is sometimes a precancerous condition; some authorities believe that "the female breast is a precancerous organ" and that carcinoma may supervene on any type of breast parenchyma.

In the present series of 800 cases, epitheliosis unaccompanied by other proliferative lesions was present in 12.9 percent; all of these were in women over 40. Epitheliosis alone occurred more frequently in fatty than in fibrous breasts. All cases of epitheliosis, alone or accompanied by other proliferative lesions, numbered 176 out of 800 cases or 22 percent. Cases occurred in the age range 26 to 85 although cases below age 41 were infrequent. There was a sharp rise from the period 36 to 40 to 41 to 45. This is of interest because the majority of clinical breast lesions are benign below age 40 and over 50 the majority are malignant. The predominance in women with fatty breasts is similar to that of pure epitheliosis. About 68 percent of all cases were mild, 25 percent moderately severe, and 6.8 percent marked. In 73.4 percent, the epitheliosis was intraductal, with intraductal and intra-acinar hyperplasia present together in 20.4 percent. Only 6.2 percent was confined to the acini. Acinar affection never occurred in women under 40. The commonest form was the papillary, seen either alone or together with other forms in 66.5 percent of all cases. Cribriform and solid forms occurred much less frequently in approximately similar incidence. Of the 12 severe cases, 3 accompanied carcinoma and 1 papillomatosis; of the 44 moderately severe cases, 6 accompanied papillomatosis, 3 occult carcinoma, and 3 clinical carcinoma; of the 120 mild cases, 6 accompanied papillomatosis, 2 clinical carcinoma, and 1 occult carcinoma. *In toto* epitheliosis accompanied papillomatosis 13 times, clinical carcinoma 8 times, and occult carcinoma 4 times.

Papillomatosis cannot always be sharply defined from papillary epitheliosis, although the highly organized papilloma is readily recognized. In the present series 10 instances of papillomatosis and 13 of true papilloma

were noted—a total of 23 cases or 2.9 percent. Of the true papillomas 4 were uniradiculate and 9 multiradiculate. Of these 10 were solitary and 3 multiple. Of all cases of papillomatosis and papilloma, 13 were associated with epitheliosis; 9 occurred in fibrous and 14 in fatty breasts.

In the 51 cases of epitheliosis and papillomatosis in which the ovaries and pituitary were examined, no consistent or constant feature was observed. The ovaries varied from highly to poorly cellular and in some cases had been previously removed. Two were the seat of granulosa-cell tumor. The pituitary picture likewise varied. Various appearances of hyperplasia or adenoma were noted, but no constant finding was present.

Epitheliosis Summary

1) Of 44 moderately severe cases, 23 were unaccompanied by other lesions;
   7 aged 40, 42, 45, 47, 52, 54, and 55 also showed adenosis;
   1 aged 58 showed sclerosing adenosis;
   1 aged 66 showed sclerosed and intermediate sclerosing adenosis;
   2 aged 64 and 75 also showed papillomatosis and sclerosing adenosis;
   1 aged 42 showed papillomatosis and adenosis;
   3 aged 47, 73, and 76 showed papillomatosis;
   3 aged 73, 76, and 77 showed occult carcinoma;
   3 aged 56, 63, and 71 accompanied clinical carcinoma.
2) Of 12 marked cases, 6 were unaccompanied by other lesions;
   1 aged 73 showed adenosis;
   1 aged 72 showed papillomatosis and 1 aged 40 sclerosed sclerosing adenosis;
   1 aged 63 showed clinical carcinoma, 1 aged 43 carcinoma and adenosis, and another aged 65 showed adenosis, clinical carcinoma, and Paget's disease of the nipple.
3) Of 120 mild cases, 78 were unaccompanied by other lesions;
   16 showed lobular adenosis—aged 29, 32, 32, 42, 46, 47, 48, 49, 55, 57, 60, 60, 60, 66, 67, and 82;
   9 showed florid sclerosing adenosis aged 55, 58, 59, 61, 63, 64, 66, 71, and 84;
   8 showed intermediate sclerosing adenosis aged 51, 51, 61, 69, 72, 75, 77, and 78;
   6 showed papillomatosis aged 51, 61 (with adenosis), 63, 69, 78, and 82 (with adenosis);
   2 showed clinical carcinoma—aged 58 (with adenosis) and 68;
   1 aged 50 showed occult infiltrative carcinoma and sclerosed sclerosing adenosis.

RELATIONSHIP OF EPITHELIOSIS WITH DUCT ECTASIA AND PERIDUCTAL ELASTOSIS

In view of the belief that retention of secretion in dilated ducts may play a part in the development of neoplasia it was obviously worthwhile to determine the coexistence of ectasia with epithelial proliferation. Further, since it has been claimed that periductal elastic hyperplasia is associated with hyperplasia of epithelium this has also been considered.

Ectasia was present to some degree in 141 of 189 cases of epitheliosis and papillomatosis, *i.e.*, in about 75 percent (but was marked only in 8 or about 4%). Some degree of ectasia or cyst formation was present in

574 of the total 800 cases studied, in about 72 percent. Of 574 cases showing ectasia or cysts epitheliosis was present in 141 cases, an incidence of 24.5 percent, not significantly different from the over-all incidence of epitheliosis for the whole series, *i.e.*, 22.0 percent.

The only evidence suggesting an influence of duct dilatation is that of 8 cases with epitheliosis associated with gross ectasia, 3 of which, 37.5 percent, showed moderate or marked epitheliosis. Of 54 with moderate ectasia and epitheliosis, the latter was moderate or marked in 9 cases, about 17 percent, and in 79 with mild ectasia and epitheliosis, the latter was moderate or marked in 13, about 17 percent. The figures are small but where ectasia was gross there may be a likelihood of more marked hyperplasia.

If we turn now to periductal elastic hyperplasia or elastosis, we find that of 189 cases of epitheliosis or papillomatosis, 29 showed periductal elastosis (gross in 5 cases only), an incidence of 15.3 percent. But of all the 800 cases studied 129 showed periductal elastosis, an incidence of 16.1 percent. The figures are very similar indeed.

Summary

In the present study no convincing evidence has been discovered of an association between duct ectasia or periductal elastosis and epithelial hyperplasia.

MAMMARY CARCINOMA

Perhaps no tumor in human pathology has attracted so much attention as cancer of the breast, with the result that a vast corpus of literature is available. Fortunately many compendious reviews have been assembled by assiduous workers and some of these may be mentioned. For those who delight in statistics the monograph of Smithers *et al.* (1952) from the Royal Cancer Hospital, London, will provide much food for thought. Although factors influencing prognosis, studies of results of treatment, and evaluation of treatment policies take pride of place, there is much to interest the pathologist also. Well-reasoned comments on such facets as heredity, social class and occupation, hormone balance, "cystic hyperplasia," trauma, marital state, parity, and breast feeding are available with many compilations of figures which betoken a great deal of literary research and re-arrangement of material. An exhaustive list of references (with full titles of all papers listed) is provided. In short this monograph serves a most useful purpose as a source book for numerical studies and references to their origin.

Lewison (1955) has also compiled a helpful book on breast cancer and its diagnosis and treatment. This is essentially a surgical treatise and considerable emphasis is laid on prognosis and treatment, but there is also an excellent historical review and interesting chapters on the anatomy and physiology of the breast. A full discussion of experimental aspects of mammary cancer in mice is contributed by Bittner; and a concise section on pathology, by Horn.

Haagensen's great monograph (1956) is also indispensable; both surgeon and pathologist will find it immensely valuable. The pathological sections show the influence of Arthur Purdy Stout, an outstanding morbid anatomist and histologist, and the photomicrographs are of a very high standard indeed. Geschickter (1945) published another useful compendium dealing with breast disease generally but containing several hundred pages on the pathology and treatment of mammary neoplasia and dysplasia and discussing fully the information on the genesis of neoplasia which was then available. The terminology may give some difficulty but there are many interesting numerical studies and photomicrographs. Saner (1950) edited a smaller British book of similar type but in this greater prominence is given to clinical aspects and the pathology of the breast is only cursorily mentioned.

For the pathologist, the large monograph of Cheatle and Cutler (1931), now unfortunately out of print, is invaluable if not indispensable. The work of these authors on epithelial hyperplasia or epitheliosis has already been discussed and it may be said that examination of whole breasts by giant sections has provided much important information, not only on the genesis but the subsequent growth and dissemination of breast carcinoma.

The section compiled by Schultz-Brauns in Henke and Lubarsch's Encyclopaedia is also valuable, despite some difficulties with terminology, for its detailed descriptions of mammary neoplasms, both anatomically and histologically. The bibliography is very full and includes many references to British and American papers as well as to Continental writers. In this respect it is a very valuable source book although the spelling of some authors' names is inaccurate. The references appear to be virtually complete up to the date of publication.

Deaver and McFarland's book (1917), although now outdated, is a mine of information on some of the more exotic abnormalities of the breast as well as the more common neoplasms.

For a short, readable, and forthright account of epithelial tumors of the breast, Willis's chapter in his important work on the pathology of tumors (1960) is to be greatly recommended. His chapters on the general nature of neoplasia, the mode of origin, structure, growth, and spread of tumors are also invaluable. Although the reader may not always be persuaded by Willis's arguments, he can scarcely fail to be stimulated and refreshed by studying them. Willis's monograph (1952) on the spread of tumors may also be perused with profit.

I have also found Stewart's fascicle (1950) on tumors of the breast in the atlas of tumor pathology of the Armed Forces Institute of Pathology to be of great value. The many illustrations are beautifully reproduced and the accompanying text is racy and provocative. An especially valuable portion deals with lesions which may simulate tumors of the breast and the description of sclerosing adenosis is very adequate.

Among individual publications, the papers of Muir (1935, 1939, 1941) are most important studies in the evolution of mammary carcinoma and of the pathogenesis of Paget's disease of the nipple, admirably illustrated and persuasive in their arguments.

Muir's paper on the evolution of carcinoma (1941) has already been considered in the preceding section. As regards the pathogenesis of Paget's disease of the nipple [*see also* Paget (1874) for his excellent clinical description of the condition which now bears his name], Muir has shown that this is the result of intra-epidermal spread of carcinoma cells which have arisen in the epithelium of the main ducts of the nipple, and Inglis (1936, 1946, 1952) has confirmed this. Toker (1961 has also illustrated most elegantly a carefully studied case of Paget's disease. However, one must point out that Willis (1960) and Cheatle and Cutler (1931) believe that Paget's disease of the nipple is a primary epidermal change and that the neoplastic change in the duct epithelium is concomitant. The paper of Willis and Goldie (1959) is also of interest in this respect. For brief reviews of Paget's disease of the male breast, reference may be made to Sandison (1956) and to Treves (1954) and for a general review of cancer in the male breast to Treves and Holleb (1955).

The works of Dawson (1932, 1933, 1934–35, 1943, 1948, 1958), Dawson and Tod (1934), Dawson and Shaw (1937), and Dawson and Davie (1942) form an exceedingly valuable series of papers, most of which are exhaustively illustrated and all of which are carefully reasoned. Dawson's paper (1954) on sclerosing adenosis has been discussed. No student of mammary pathology can afford to ignore Mrs. Dawson's long series of scholarly publications.

In the preceding section dealing with epitheliosis and papillomatosis reference was made to work on the relationship between epitheliosis and the development of neoplasia. Charteris (1930) studied the changes in the mammary gland preceding carcinoma. He believes that the changes, which he describes as progressive epithelial hyperplasia, are in reality only different stages of one process that finally ends in malignancy. His findings are based on detailed examination of 48 breasts removed for carcinoma, and confirm those of Muir in many particulars. Godwin (1952) described a case of intra-acinar carcinoma. The patient had a biopsy in 1935, the carcinoma later disseminated and radical mastectomy was done in 1948. She finally died and was autopsied in 1950.

I also refer briefly to the papers of Forrest *et al.* (1956, 1958, 1959) on radon implant of the pituitary for advanced breast carcinoma, since some of the cases so treated are considered in passing here. With reference to the need for early diagnosis of carcinoma in younger women, I (1955) cited that in 301 specimens of breast from women under age 40 no fewer than 58 proved to be carcinoma.

With regard to the opposite or remaining breast in women who have had cancer of one breast, Fitts (unpublished work cited by Horn in Lewison 1955) found that in 724 breast cancer patients 66 showed bilateral disease, an incidence of 9 percent. Of these, 12 came under observation after previous treatment for carcinoma, 13 had simultaneous bilateral carcinomas, and 41 developed their second carcinoma while under follow-up observation after treatment of the first. It was not possible to say how many of these were second primaries and how many represented primary tumors. Foote and Stewart (1945) stated that "the most frequent precancerous lesion of the breast is a cancer of the opposite breast." Kilgore (1921) claimed that the expectancy of carcinoma in the second breast of women who survived more than 5 years after radical mastectomy for carcinoma was about 5 to 7 percent, and he advocated that, if the axillary nodes were uninvolved in the radical mastectomy specimen, the second breast should be resected also by simple operation. More recently Guiss (1954) claimed that incidence of bilateral involvement is less than 5 percent and that prophylactic resection of the uninvolved breast is not justified. Hubbard (1953) asserted that of 264 patients who had carcinoma of the breast 8 or 3.4 percent developed proven carcinoma in the opposite breast. He resected the opposite breast of 16 patients and in 2 discovered small foci of unsuspected carcinoma. Farrow (1956) has also discussed bilateralism of mammary cancer. Mider *et al.* (1952) in a paper on multiple cancer noted that, of 941 cases of breast cancer, 59 showed multiple neoplasms, an incidence of 6.3 percent. Qualheim and Gall (1957) in a study of 157 breasts removed for carcinoma found in whole-organ sections that about 50 percent showed multiple independent foci. Willis (1952) points out that carcinoma of the opposite breast may be due to extension from the primary tumor by lymphatic emboli or by permeation, but that some cases of bilateral tumors are the result of multicentric bilateral simultaneous origin. I have discussed this problem at a little length because the main value of the numerically small series, which I now detail, lies in the study of the remaining breast.

Results

In all, 25 cases were studied in which mastectomy had been carried out, but 1 of these was a mammary malignant melanoma and may more properly be excluded from our consideration, except to mention that the remaining breast did not show secondary melanoma, but only mild intraductal cribriform epitheliosis. The remaining 10, which were clinically detected, had not had mastectomy, 3 were untreated, and the remaining 7 had different forms of therapy. Six cases of occult carcinoma were discovered in this study. In all, 40 cases are reviewed briefly in this section.

In the morphology of these tumors no fact of great interest emerges. No instance of colloid carcinoma was studied and the majority of the neoplasms were of the ordinary infiltrative spheroidal cell type eliciting a more or less scirrhous reaction. However, one of the occult carcinomas was of distinctly adenocarcinomatous pattern, and another was of intraductal comedo type only. In 2 cases, in which mastectomy was not done, there was infiltrative and intraductal carcinoma and one of these had

Paget's disease of the nipple. The carcinomas in the opposite breast were each, in my opinion, secondary and not the result of bilateral spontaneous origin. They were all infiltrative tumors, of appearance entirely consistent with spread from the primary site.

In none of the cases in which the pituitary had been more or less severely damaged by radon implant was there any appreciable difference in the appearance of the tumor cells either in primary or metastatic sites. In no case, however, was complete destruction of the hypophysis achieved. (Subsequent cases in which total destruction has been achieved have not shown marked changes in cell morphology.)

Of the 34 clinically detected cases the primary tumor was on the right side in 17 and on the left in 17. It is usually stated that carcinoma is more common on the left side, possibly because in right-handed persons the left breast is more prone to trauma than the more protected right breast. The breast types concerned were fibrous in 15 and fatty in 19. In 1 case the patient also had carcinoma of the bronchus and 1 other had acute lymphatic leukemia.

Considering the 24 cases in which mastectomy had been done, the longest survival recorded was 6 years. One patient had radiotherapy alone after mastectomy; 1 other had radiotherapy followed by radon implant of the pituitary; 2 others had radiotherapy and hormone therapy followed by radon implant of the pituitary; and 3 others had radon implant only. Secondary carcinoma was seen in the remaining breast in 7 patients (accompanied in 1 by adenosis while the remaining 6 showed an atrophic parenchyma)—an incidence of 29.2 percent. Atrophy or simple cyst formation only was seen in 7 cases, an incidence of 29.2 percent. Adenosis, apart from the case accompanying secondary carcinoma, was seen in 4 others, the total incidence being 20.8 percent. Sclerosing adenosis was noted in 2 cases (in 1 this accompanied epitheliosis and papillomatosis), an incidence of 8.3 percent. Epitheliosis was present in 5 (accompanied in 1 by papillomatosis and sclerosing adenosis), an incidence of only 20.8 percent; of these 2 were moderately marked and 3 were mild. The site of the epitheliosis in 4 was confined to the ducts and in 1 was present in ducts and acini. The pattern of epitheliosis was predominantly papillary in 4 cases and cribriform in 1. If we disregard the presence of secondary carcinoma the breast parenchyma was atrophic in 13 cases, an incidence of 54.2 percent.

If we turn now to the 10 cases in which mastectomy was not performed, we find that carcinoma was still present in the breast of primary origin in 9 cases. Only in 1 woman who had had radiotherapy were both breasts completely atrophic; nevertheless, she had visceral carcinomatosis and had had a pituitary radon implant. In the remaining 9 cases, carcinoma was still present in the primary breast site although intraductal origin could only be detected in 2 and was present in the opposite side in 3 (fig. 26). The treatment used was symptomatic only in 3 instances: radiotherapy alone in 3 cases; radiotherapy followed by pituitary radon implant in 1; pituitary radon implant only in 1; and oophorectomy and adrenalectomy in the remaining 2. The 2 latter cases were those in which the tumor appeared in pregnancy: There was carcinoma in both breasts; adenosis was noted in both; and in 1 there was gross epitheliosis in each breast also. In 6 cases there was epitheliosis in the primary breast (2 mild, 2 moderately severe, and 2 gross) and in 4 there was also epitheliosis in the opposite breast. In the exceptional case the epitheliosis was mild on the primary side, while the opposite was atrophic. In 1 case, however, the epitheliosis was mild on the primary side and gross on the opposite side. In another case there was no apparent epitheliosis on the primary side but gross epitheliosis on the opposite side. In all cases the epitheliosis on the primary side was sited in ducts, but on the opposite side in 2 instances the acini were also affected. The pattern of proliferation was either papillary or solid in most instances. It is possible that in the latter 2 cases the presence of primary carcinoma may have obliterated pre-existent epitheliosis. If we now consider the opposite side only we see that secondary carcinoma was present in 3 cases, accompanied in 2 by epitheliosis, and in 1 by adenosis. In 3 the opposite breast showed atrophy only and in 1 florid sclerosing adenosis. In 5 the opposite breast showed epitheliosis which was moderate in 2 and gross in 3 instances. Paget's disease of the nipple was present in 1 instance on the primary side.

Occult carcinoma was present in 6 of the 776 cases in the series which were not clinical carcinomas, an incidence of 0.77 percent. Of these 6 cases, 5 showed infiltrative carcinoma only and 1 intraductal carcinoma (fig. 25). The latter was accompanied by sclerosing adenosis. The remaining 5 cases were accompanied by epitheliosis which was mild in 1, moderate in 3, and gross in 1. In each case ducts only were affected and there was an even distribution of types. As regards the opposite breasts, 3 showed atrophy and 2 epitheliosis, 1 gross and 1 mild. The mild epitheliosis affected both ducts and acini. Both showed solid areas with cribriform pattern in one and papillary in the other.

It is interesting to consider the age at death of these cases: Of the 6 with occult carcinoma 5 were in the eighth decade, and the 6th was age 50. Occult carcinoma of the prostate appears to be present in old men in proportion to their actual age, *i.e.*, is present in a higher percentage of cases at autopsy in each succeeding decade. Of the clinical cases, scattered over the period 30 to 84 years, 6 were less than 40 (17.6% of all cases) and 11 under age 50 (32.7% of all cases). These figures may be compared with my surgical series: Of 540 carcinomas, 40 occurred under age 40 (7.4%) and 179 under age 50 [33.1%, (1958)].

We may consider together the opposite breasts of all the 40 cases of carcinoma studied. Secondary spread had occurred in 10 of the 34 clinical cases, *i.e.*, in 29.4 percent, and since there was no secondary spread in the cases of occult carcinoma, the over-all incidence in all carcinomas falls to 25.0 percent (fig. 27). The parenchyma of the opposite breast was atrophic in 16 of the 34 clinical cases and in 19 of the total 40 cases, in 47.5 percent. Adenosis was noted in only 9 of the 40 studied, *i.e.*, in 22.5 percent, and epitheliosis in 12 opposite breasts of the 40 cases or

30 percent. This was mild, moderately severe, and severe in 4 instances each.

Regarding the ovaries in the 21 cases in which the gonads and endocrine organs were studied we find that 4 women, the oldest 43, had had therapeutic oophorectomy. Of the remaining 17 secondary carcinoma was present in 4 ovaries, 23.5 percent, and there were leukemic deposits from a coexistent lymphatic leukemia in another. A small Brenner tumor was present in another ovary. Only 2 cases (aged 33 and 40) showed continuing follicular activity. Ten cases (58.8%) had a highly cellular (+ + +) stroma, 4 (23.5%) a moderately cellular (+ +), and 3 (17.7%) a poorly cellular (+) stroma. Of the latter 3, however, it should be mentioned that 2 had had massive pituitary necrosis induced by radon implant.

No consistent ovarian picture emerges, but the proportions of stromal cellularity are rather less frequent in the higher cellularity groups than in the over-all series (*see* section on Endocrine Relationships).

With regard to the pituitary, secondary carcinomatous deposits were present in 6 cases, 28.6 percent, and extracapsular leukemic deposits in 1 case of coexistent lymphatic leukemia. In 9 the pituitary had been implanted with radon, producing much necrosis in 5 and little necrosis in 4. Of the unaffected pituitaries 4 appeared entirely normal; in 1 a chromophobe adenoma was present and in 2 the chromophobes were rather prominent; fetal-type chromophobes were noted in 3. Beta cells appeared diminished in 2 and alpha cells were prominent in 1. Again it will be seen that no consistent pattern emerged.

The adrenals had been therapeutically removed in 4 women, the eldest aged 43. In 12 cases secondary carcinoma was noted in 4, *i.e.*, in 33.3 percent and leukemic deposits in 1 case of coexistent lymphatic leukemia. Medullary hyperplasia was viewed in 3 and cortical adenomatous hyperplasia in 1 case. In 5 cases no abnormality of any sort was detected.

The thyroid was examined in 10 cases and no abnormality was detected in 7 of these. In 1 there was diffuse secondary carcinoma, in 1 a Hürthle-cell adenoma, and in another simple adenomas.

The pancreas was examined in 9 cases, but there was no evidence of any abnormality.

Summary

Twenty-four cases of breast carcinoma in which radical mastectomy had been performed, 10 cases in which no operative removal of the tumor had been attempted, and 6 cases of occult breast cancer were studied. None of these showed any unusual morphology either in primary or metastatic sites. Most were infiltrative spheroidal cell carcinomas: 2 showed also intraductal carcinoma, 1 with Paget's disease of the nipple.

In this series the right and left breasts were equally affected: fibrous in 15 and fatty in 19. In 1 there was also carcinoma of the bronchus and in another acute lymphatic leukemia. The treatment given in the clinically detected cases varied greatly and included surgery, radiotherapy, hormone therapy, oophorectomy, adrenalectomy, and attempted hypophyseal destruction. Carcinoma was present in the opposite breast in 10 of the 40 patients studied, *i.e.*, 25 percent; in the ovary in 4 of 17, 23.5 percent; in the pituitary of 6 of 21, 28.6 percent; in the adrenals of 4 of 12, 33.3 percent; and in the thyroid of 1 of 10, 10 percent.

Epitheliosis of primary origin was in the breast in 10 of 16 cases available for study or in 63 percent. The opposite breast was studied in all 40 cases (excluding the presence of secondary carcinoma) and was atrophic in 47.5 percent, showed adenosis in 22.5 percent, and epitheliosis in 30.0 percent of all cases.

The stromal cellularity of the ovaries was frequently high, but no consistent appearance was seen throughout in either ovaries or pituitary gland.

APPEARANCE IN BREAST, WITH MALIGNANT NEOPLASIA IN OTHER ORGANS

A short analysis has been made of the number of cases of malignant neoplasm, of primary site other than breast, associated with proliferative change in the breast (tables 23, 24, and 25).

Carcinomas have been divided into four groups (*a*) alimentary, (*b*) respiratory, (*c*) genital, and (*d*) urinary. None of 7 cases of urinary carcinoma showed proliferative breast change. About 45 percent of cases of alimentary and respiratory carcinomas showed proliferative change. This is about the average for the whole group. With genital carcinoma

TABLE 23.—Malignant tumors other than breast carcinoma

	Carcinoma							
	Alimentary		Respiratory		Genital		Urinary	
Age group	Number of cases	Number showing breast proliferation	Number of cases	Number showing breast proliferation	Number of cases	Number showing breast proliferation	Number of cases	Number showing breast proliferation
21–25	—	—	—	—	—	—	—	—
26–30	—	—	—	—	—	—	—	—
31–35	2	0	—	—	1	0	—	—
36–40	—	—	1	0	2	2	—	—
41–45	1	1	—	—	3	1	—	—
46–50	3	1	4	2	1	1	—	—
51–55	4	3	5	3	1	1	1	0
56–60	4	2	1	0	1	0	2	0
61–65	15	7	3	1	6	3	3	0
66–70	15	5	4	3	2	1	—	—
71–75	15	6	5	2	1	1	1	0
76–80	4	3	—	—	—	—		
81–85	2	2	—	—	—	—		
86–90	1	0	—	—	—	—		
21–90	66	30(45.4%)	23	11(47.8%)	18	10(55.5%)	7	0

TABLE 24.—Malignant neoplasms associated with proliferative change in the breast

	Carcinoma					
	Alimentary	Respiratory	Genital	Urinary	Melanoma	All sarcoma group
Total number of cases	66	23	18	7	2	32
Secondary carcinoma	1	2	0	0	0	0
Clinical breast carcinoma	1	0	0	0	0	1
Occult breast carcinoma	1	0	1	0	0	0
All epitheliosis	17	4	6	0	0	4
Epitheliosis only	13	2	3	0	0	2
All adenosis	8	4	4	0	0	4
Adenosis only	6	1	3	0	0	2
All sclerosing adenosis	7	4	3	0	0	0
Sclerosing adenosis only	1	2	1	0	0	0
All papillomatosis	1	1	0	0	0	1
Papillomatosis only	0	1	0	0	0	0
Fibroadenoma	1	0	0	0	0	0
Deposits of Hodgkin's disease	0	0	0	0	0	2
Deposits of leukemia	0	0	0	0	0	3
Deposits of reticulosis	0	0	0	0	0	1

55.5 percent showed breast proliferation. The numbers involved are small but at least the trend indicates some possible hormonal influence affecting both genitalia and breast. I feel this question might be pursued advantageously. Epitheliosis is present in 17.4 percent of respiratory carcinomas, 25.8 percent of ailmentary carcinomas, and 33.3 percent of genital carcinomas. The figures are probably not significant statistically, but not inconsistent with some link between genital carcinoma and breast change. Adenosis is also present in a rather higher proportion of genital carcinomas than in the others.

As regards secondary neoplasm in the breast 1 case of secondary pancreatic carcinoma, 2 secondary bronchial carcinoma, 2 Hodgkin's deposits,

TABLE 25.—Leukemias, Hodgkin's disease, and reticulosis

Age	Case	Presence of deposits
44	Chronic myeloid leukemia	No
55	Acute leukemia	Yes
64	Monocytic leukemia	No
60	Chronic myeloid leukemia	No
30	Acute lymphatic leukemia	Yes
46	Subacute myeloid leukemia	Yes
30	Hodgkin's disease	Yes
56	Hodgkin's disease	Yes
31	Hodgkin's disease	No
60	Acute histiocytic medullary reticulosis	Yes
48	Malignant lymphoid neoplasm	No
55	Lymphosarcoma	No
56	Lymphosarcoma	No
61	Lymphosarcoma	No
62	Lymphosarcoma	No
51	Reticulosarcoma	No
55	Reticulosarcoma	No
42	Multiple myelomatosis	No
62	Mycosis fungoides	No
69	Mycosis fungoides	No

3 leukemic deposits, and 1 with deposits of reticulosis (acute histiocytic medullary reticulosis) were noted (figs. 28 through 32).

Table 25 tabulates all cases of leukemia, Hodgkin's disease, reticulosis, and reticulo- or lymphosarcoma, etc. It will be seen that the 2 cases of Hodgkin's disease which affected the breast constitute two thirds of all cases; that the reticulosis case was the only one affecting the breast in a group of 8; that 3 of 6 leukemias affecting the breast were of acute type. One case of multiple myelomatosis and 2 of mycosis fungoides did not affect the breast.

For a brief account of metastatic tumors in the breast, reference may be made to Willis (1952). He cites instances of deposits of lymphatic leukemia; round-cell tumor, possibly of ovary; carcinoma of thyroid; stomach; and parotid gland. Dawson (1936) also reviewed metastatic breast tumors and concluded that they are rare. She reported a case of gastric carcinoma producing bilateral mammary carcinosis. Geschickter (1945) devoted a section of his book to lymphosarcoma and "myelosarcoma" of the breast. In a survey of 301 breasts of younger women I found (1955) instances of mastectomy for lymphosarcoma and hemic neoplasm, and I have further discussed metastatic breast tumors found surgically and at autopsy (1959).

Seifert (1952) has written an interesting paper on "Tissue Reactions in the Human Mammary Gland in Leukemia," and suggests that in some leukemias, especially in acute myeloblastic disease, myelopoiesis may be seen in the breast, more often in young women and possibly in some way associated with pregnancy.

Haram (1937) described a case of lymphatic leukemia with bilateral mammary masses treated by mastectomy.

Of the 3 cases noted in the present series, the first was a 55-year-old woman with acute leukemia who died, undiagnosed, a few moments after admission. The marrow showed acute leukemic hyperplasia of largely blast-cell type. Breasts showed periductal infiltrates of large cells with numerous mitoses in both sides. In the second case, a 46-year-old woman died soon after abdominoperineal resection of the rectum and at autopsy had subacute myeloid leukemia with deposits in liver and spleen. There were leukemic cells in the lobules of the breast, eosinophil polymorph precursors being very readily seen. The third case, a woman of 30, had had a radical mastectomy for breast carcinoma. At autopsy she displayed acute lymphatic leukemia with changes in bone marrow, spleen, liver, heart, ovaries, adrenal and pituitary capsule. The remaining breast showed diffuse leukemic large cells in fibrous stroma and in the lobules.

Summary

The appearance in the breast has been analyzed in malignant neoplasms, other than breast carcinoma. The only possible significant relationship is the relatively high incidence of all proliferative lesions and epitheliosis in women with genital carcinoma. Leukemic and Hodgkin's disease deposits have been noted.

CONDITION OF BREAST WHEN DEATH RESULTED FROM OTHER THAN NATURAL CAUSES

It seems worthwhile to consider these cases together to see if the appearance in the breast is grossly different from the over-all picture, since it may be argued that the lesion producing death might materially alter the condition of the breast.

In the present series of 800 cases, 15 women died as the result of trauma and 10 of poisoning (barbiturates being the most common substances). In these cases there was no gross pathology. Of the 15 women who died from trauma, 8 had atrophic parenchyma, an incidence of 53.3 percent which corresponds well with the over-all average. Six women showed epitheliosis, an incidence of 40 percent, and 5 adenosis or sclerosing adenosis, *i.e.*, 33.3 percent. These figures are small but the incidence of epitheliosis is rather higher than the average rate, and it is theoretically possible that protracted disease before death lessens the probability of women showing epitheliosis. As regards the 10 cases of poisoning, 3 only showed atrophy, 30 percent, while 3 showed epitheliosis, and 4 adenosis. Here the proportion of proliferative changes is rather higher than usual.

TABLE 26.—Breast conditions in accidental or poisoning deaths

Age group	All cases	Atrophic	All adenosis or sclerosing adenosis	All epitheliosis/ papillomatosis	All proliferative lesions
21–25	—	—	—	—	—
26–30	1	—	1	1	1
31–35	1	—	1	—	1
36–40	2	1	1	—	1
41–45	1	—	1	—	1
46–50	1	1	—	—	—
51–55	4	1	1	2	3
56–60	1	—	1	—	1
61–65	3	2	1	1	1
66–70	4	3	—	1	1
71–75	2	1	1	1	1
76–80	3	1	1	2	2
81–85	2	1	1	1	1
86–90	—	—	—	—	
21–90	25	11 (44%)	10 (40%)	9 (36%)	14 (56%)

Considered together these constitute 25 cases, of which 11 showed atrophy (44%) and 14 proliferative change (56%); of these, 10 showed adenosis or sclerosing adenosis (40%) and 9 epitheliosis or papillomatosis (36%).

The numbers are fairly small but as far as they go they suggest that women dying without gross disease processes may be slightly more likely to show proliferative changes, especially epitheliosis (table 26).

APOCRINE METAPLASIA

For a brief annotation on the apocrine glands reference may be made to the *British Medical Journal* of May 22d, 1954. The presence of apocrine-like epithelium in the breast has for many years been recognized, and Lendrum (1945) gives a review of the long-continued dispute as to whether it represents a sweat gland remnant or a secondary alteration in the mammary epithelium. He describes an excellent method for demonstrating granules in this "pale" epithelium, and, by analogy with islets of intestinal epithelium in the gastric mucosa, opines that the occurrence of this phenomenon represents a metaplasia or a regeneration following dedifferentiation to a more primitive type. I find Lendrum's arguments persuasive, and I believe that this is a metaplasia.

Dawson (1932) wrote a critical account of the origin of "pale" epithelium in the breast and also concluded that it was derived from mammary tissue. She found no evidence that "sweat gland carcinoma of the breast" ever occurred, and with this view I also concur. Nevertheless, some American authors, *e.g.*, Stewart (1950) and Geschickter (1945) believe that about 1 percent of mammary carcinomas are "apocrine sweat gland carcinomas" and Ewing (1940) went so far as to claim that a considerable proportion of adenocarcinomas of the breast were of sweat gland origin.

Berning and Bücker (1937) investigated the relationship between "Apokrine Drusencysten und Carcinom" and found no significant relationship. Foote and Stewart (1945) found apocrine metaplasia in 42 percent of 200 noncancerous breasts and in 39 percent of 300 cancerous breasts, most frequently in the decade 40 to 50. As previously stated, Stewart concluded that 1 percent of mammary cancers originated in apocrine epithelium. Frantz *et al.* (1951) in their study of postmortem breasts found apocrine cells in 107 of their 225 cases, an incidence of 50.7 percent, often associated with hyperplasia of the epithelium.

Results

I found apocrine metaplasia in 39.2 percent of my 800 cases. The maximum incidence was in the period 56 to 85 years, though instances were noted in all periods. These included: 1 instance in a papilloma, 1 in a frank epitheliosis, and 3 in areas of sclerosing adenosis.

Although in many instances the cysts lined by "pale" or "pink" epithelium showed some papillary ingrowths, I do not believe that apocrine metaplasia ever becomes frankly proliferative or neoplastic (table 27, and figs. 33 and 34).

Summary

"Apocrine metaplasia" is, as the name I have chosen implies, a metaplasia of the mammary epithelium. It is seen frequently in postmortem breasts, especially in older women. It may occur in papilloma, epitheliosis, or sclerosing adenosis but is not in itself a frankly proliferative lesion.

TABLE 27.—Apocrine metaplasia*

Age group	Number of cases	Percent of all cases
21–25	1	7. 7
26–30	1	8. 3
31–35	6	13. 7
36–40	11	33. 3
41–45	13	28. 8
46–50	17	29. 3
51–55	34	34. 8
56–60	43	45. 7
61–65	55	39. 8
66–70	61	53. 5
71–75	43	44. 3
76–80	20	54. 0
81–85	8	66. 6
86–90	1	20. 0
21–90	314	39. 2

*These include 1 case with apocrine change in a papilloma, 1 in epitheliosis, and 3 in sclerosing adenosis.

PERIDUCTAL "ELASTOSIS"

The condition of diffuse hyperplasia of the elastica of the ducts and even of the acini of the breast was described by Cheatle and Cutler in their monograph (1931). They state that the amount of hyperplasia may be very great and may consist of up to one hundred layers of elastic fibers. The epithelium of the ducts may be normal, hyperplastic, or even neoplastic. Cheatle and Cutler believe that elastosis possibly indicates that some other pathological process is occurring in the gland, other than an adenosis phenomenon. Schultz (1933) in Henke and Lubarsch's Encyclopaedia depicted marked periductal elastic hyperplasia as part of the phenomenon of senile involution, often associated with duct ectasia and sometimes obliteration of the ducts. On the other hand, Jackson and Orr (1957) believe that elastosis and subepithelial-intraelastic-collagen increase may have a precancerous significance. Emerson *et al.* (1953) showed that marked elastic hyperplasia occurred around the ducts of 13 of 79 cases of breast cancer untreated by radiotherapy or hormone administration.

Muir and Aitkenhead (1934) described 2 cases of Paget's disease of the nipple in which the associated intraductal neoplasia had at places undergone regression by reactive change in the intraelastic connective tissue which underwent hyperplasia. In some instances the obliterated ducts were ultimately represented by hyalin material, avascular and acellular surrounded by a ring of abundant elastic tissue.

More recently Lumb (1950) described the changes in the breast following irradiation for carcinoma and noted elastic tissue as a diffuse network in the masses of debris where neoplastic cells had previously existed and also around the ducts.

In the present study this process of periductal elastic hyperplasia was frequently seen. In some instances the layers of elastic tissue were clearly discrete and separate, but, in other areas in the same breast, the appearance was that of an irregular network or even an amorphous-looking mass.

Elastosis or elastic hyperplasia was noted in 129 of 800 cases, an over-all incidence of 16.1 percent. The condition is more common after age 50 although it is occasionally seen in younger women. When it occurs in women under 56 it is never marked, although after that age it occasionally may be (5 or 38.7% of all cases of elastosis) and is sometimes quite obliterative (in 14 or 10.8% of all cases of elastosis). One other case of noticeable obliterative change was noted after radiotherapy, but in general this was not a marked result of previous treatment. *See* table 28, and figures 35 and 36

Table 28.—Periductal elastosis

Age group	Number of cases	Percent of all cases	These include: Marked	Obliterative	Marked radiational
21–25	—	—	—	—	—
26–30	1	8. 3	—	—	—
31–35	3	6. 8	—	—	—
36–40	0	—	—	—	—
41–45	2	4. 4	—	—	—
46–50	5	8. 6	—	—	—
51–55	14	14. 3	—	—	—
56–60	19	20. 2	1	2	—
61–65	27	19. 5	3	4	—
66–70	28	24. 4	1	5	1
71–75	15	15. 4	—	1	—
76–80	10	27. 0	—	1	—
81–85	4	33. 3	—	1	—
86–90	1	20. 0	—	—	—
21–90	129	16. 1	5	14	1

Summary

Some degree of periductal elastic hyperplasia is present in about 16 percent of all autopsies. It is more common in older women and is never of marked degree under the age of 56. In older women it may be conspicuous or even produce considerable obliteration of the ducts.

CYSTIPHOROUS CHANGE WITH COLOSTRUM CORPUSCLES

The formation of cysts, from the walls of which large foamy cells desquamate, has been described by many authors including Schultz (1933), Cheatle and Cutler (1931), and others reporting on findings in surgical material. At present this formation of "colostrum corpuscles" (as these large foamy cells are often called) is considered insignificant in neoplasia. Walker and Sandison (1958) could occasionally demonstrate colostrum corpuscles in nipple discharges from patients with simple fibrocystic disease.

Frantz *et al.* (1951), in a postmortem study of 225 women, showed that there were foamy cells in 25 of 42 cases which displayed marked cyst formation (59.5%), in 19 of 54 with marked ectasia of ducts (35.2%), and finally in 5 of 19 with microcysts only.

In the present series of 800 cases colostrum corpuscles were noted in 72 instances, an over-all incidence of 9.0 percent. The age incidence shows a spread over the period 36 to 85. There were no significant relationships (table 29, and figs. 37 and 38).

Table 29.—Cystiphorous change with colostrum corpuscles

Age group	Number of cases	Percent of all cases
21–35	—	—
36–40	4	12. 1
41–45	3	6. 7
46–50	7	12. 1
51–55	10	10. 2
56–60	12	12. 6
61–65	13	9. 4
66–70	9	7. 8
71–75	9	9. 3
76–80	2	5. 4
81–85	3	25. 0
86–90	—	—
21–90	72	9. 0

Summary

Desquamation of foamy cells (colostrum corpuscles) was noted in 9.0 percent of all 800 cases examined. This phenomenon is not regarded as pathologically significant.

PERSISTENT SECRETORY CHANGE IN LOBULES

This interesting phenomenon was seen frequently in women when the majority of breast lobules showed marked involutional change. It is manifested by the appearance of persistent vacuolation at the free border of cubical or columnar cells lining dilated acini. Usually only occasional lobules show the persisting secretory change, and sometimes only one may be affected.

In Henke and Lubarsch's Encyclopaedia, the condition is briefly discussed by Schultz (1933), and he depicts secretory change in a woman whose last confinement had been 19 years previously. Dawson (1954) pointed out that, in older women, involution after pregnancy and nonlactation appears to be slow. Frantz *et al.* (1951) showed that, in their 225 postmortem cases, persistent secretory cells occurred in 7 women (3.1%) whose ages ranged from 33 to 72. In the 72-year-old woman the last pregnancy had occurred 48 years before, and, except in 2 women aged 33 and 34, the ovaries showed no follicular activity.

In the present series persistent secretory change was present in 30 of 800 cases, or in 3.7 percent of all cases, and was persistent from the age group 21 to 25 until 71 to 75, more common in younger women and not seen above age 75 (table 30, and fig. 39).

TABLE 30.—Persistent secretory change in lobules

Age group	Number of cases	Percent of all cases
21–25	3	23. 0
26–30	1	8. 3
31–35	8	18. 2
36–40	3	9. 1
41–45	5	11. 1
46–50	3	5. 2
51–55	1	1. 0
56–60	—	—
61–65	2	1. 5
66–70	3	2. 6
71–75	1	1. 0
76–90	—	—
21–90	30	3. 7

Of 10 cases in which the gonads and pituitary were studied, 1 woman aged 40 had had an oophorectomy in the course of a Wertheim's hysterectomy for carcinoma of the cervix. Of the remaining 9, the stroma was + + + cellular in 7 and + + cellular in 2. Four women aged 22, 42, 47, and 50 showed continuing follicular activity in the ovaries. Hilus interstitial cells were noted in 3. The pituitary was apparently unexceptional in 3 cases, showed patchy acute necrosis in 1, a chromophobe adenoma in 1, a Rathke's cyst in 1, and predominance of chromophobes, alpha cells, or beta cells in 1 case each.

Summary

Persistent secretory change in isolated lobules was seen in 3.7 percent of all cases examined throughout the ages 21 to 75; it was commoner in younger women. Follicular activity was present in the ovaries in only 4 of 9 cases. Hilus interstitial cells were seen in 3. Most ovaries had a highly cellular stroma. The pituitary did not show any constant appearance.

ROUND-CELL INFILTRATES IN BREAST

The appearance of round cells in the breast, especially in the lobular connective tissue, is frequent and it is probable that in younger women this is part of the cyclical response of the breast to hormonal influences.

Frantz *et al.* (1951) in their examination of 225 women at autopsy found that lymphocytes were present in the breasts of 12 of 42 cases showing gross cysts; 19 of 54 showing ectasia of ducts, and in 11 of 33 showing microcysts. They do not state the over-all incidence for the 225 women examined.

In my series lymphocytes only were noted in 168 of 800 cases examined—an over-all incidence of 21.0 percent—and lymphocytes and plasma cells in a further 18, an over-all incidence of 2.2 percent (table 31). If all cases are considered together the total incidence is 23.2 percent, *i.e.*, in nearly one quarter of all cases.

Lymphocytes only were present in women whose ages ranged from 26 to 30 until the period 86 to 90. The highest quinquennial incidence was 26 to 30 years; thereafter the differences from period to period were not great. Plasma cells were relatively uncommon, but considerably more frequent in women under 45 (fig. 40).

TABLE 31.—Round-cell infiltrates

Age group	All cases showing lymphocytes only		All cases showing lymphocytes and plasma cells	
	Number	Percent	Number	Percent
21–25	—		3	23. 1
26–30	5	41. 7	1	8. 3
31–35	10	22. 8	4*	9. 1
36–40	9	27. 3	—	—
41–45	12	26. 6	4	8. 8
46–50	16	27. 6	—	—
51–55	17†	17. 3	2‡	2. 0
56–60	20	21. 2	—	—
61–65	21	15. 2	2	1. 5
66–70	30	26. 3	—	—
71–75	17	17. 5	2	2. 1
76–80	8	21. 6	—	—
81–85	2	16. 6	—	—
86–90	1	20. 0	—	—
21–90	168	21. 0	18	2. 2

*One of these women was pregnant.
†Polymorphs also noted in 1 case.
‡Eosinophils also noted in 1 case.

I do not regard the presence of plasma cells in the lobules as pathologically important. No case of plasma-cell mastitis [Haagensen (1951) and Gershon-Cohen and Ingleby (1952)] was seen in the present study.

Summary

Lymphocytes (with or without plasma cells) are frequent in the postmortem breast. In the present series 23.2 percent of all women showed lymphocytes, usually intralobular or periductal. No plasma-cell mastitis was seen.

PACINIAN CORPUSCLES

The sensory terminal apparatus in the breast is composed of a connective-tissue capsule of variable thickness surrounding the nerve ending, known as pacinian corpuscles or corpuscles of Vater-Pacini and regarded as pressure receptors (Maximow and Bloom 1948, and Ham 1953). They are probably more frequent in the superficial portions of the breast and are in fact infrequently seen in the parenchyma. In the present series of 800 cases, pacinian corpuscles were seen only in 19 instances—an over-all incidence of 2.4 percent (fig. 41).

For an interesting paper on the nerve supply of the nipple, which reviews the innervation of the breast and the presence of nerve endings, reference may be made to Cathcart, Gairns, and Garven (1948).

MAMMALITHIASIS

This term implies the presence of laminated calculus-like bodies in the ducts or acini of the breast. The bodies are of the "calcospherite" type and are usually hematoxyphilic. They do not appear to be associated with marked duct ectasia. I have not seen them frequently in either surgical or postmortem material.

Frantz *et al.* (1951) claim to have noted this phenomenon in 10 of their 225 cases. In 4 there was gross cyst formation and in 5 cystiphorous formation of foamy colostrum corpuscles.

I noted mammalithiasis in only 4 out of 800 patients, an over-all incidence of 0.5 percent, aged 36, 69, 70, and 70. All concretions were laminated: 3 were hematoxyphilic; 1, intraductal; and 3, intra-acinar in type. One of the 4 cases was associated with epitheliosis; 1 with epitheliosis and sclerosing adenosis; 1 with adenosis; 1 was present in atrophic parenchymatous ducts; the others were acinar in type (fig. 42).

Summary

Mammalithiasis was rarely seen in the present survey, the over-all incidence being 0.5 percent.

FAT NECROSIS

Some destruction of fat with phagocytosis and foreign-body giant-cell reaction may occur in the breast in a variety of conditions: following trauma, abscess, or associated with neoplasia. Fat necrosis occasionally may be confused clinically with carcinoma, and radical mastectomy has been carried out as a result. Not unnaturally, the pendulous, obese breast is more prone to fat necrosis than the other types.

In my surgical series of 1,010 female breasts 11 or about 1 percent showed lipophagic reaction. These women were in the fourth to the seventh decades (1958).

Lipophagic reaction was noted in only 2 cases, aged 59 and 70, or in 0.25 percent in the postmortem series. The former had a fibrofatty breast parenchyma while the latter was adipose; histologically both breasts were atrophic.

CONDITION OF BREAST IN SOME ENDOCRINE DISORDERS

The histological appearance of the breast in certain well-defined disorders has been analyzed: diabetes mellitus, hepatic cirrhosis, and hyperostosis frontalis interna; there are a small number of other major endocrine disorders and these are also regarded briefly.

If we consider first diabetes mellitus, we find that in the series of 800 cases, a total of 37 women died as a result of this disease; their ages ranged from 21 to 25 to 71 to 75. Of these 37 women, 8 (21.6%) showed epitheliosis, 11 (29.7%) adenosis, and 1 each (2.7%) sclerosing adenosis and papilloma. None of these incidences is unusual; the over-all incidence of epitheliosis in 800 cases was 22 percent and adenosis 22.5 percent. On the present evidence there is no reason to believe that the breast is in any way peculiar in diabetes mellitus.

We turn next to hyperostosis frontalis interna (Moore 1955, and Henschen 1949), which has frequently been alleged (not always on very concrete grounds) to be the result of endocrine disturbance. In the present series of 800 cases, 50 women showed hyperostosis; of these 26 percent showed epitheliosis, 12 percent adenosis, and 10 percent sclerosing adenosis. One case each was associated with papillomatosis, occult carcinoma, and clinical carcinoma. The slight increase in epitheliosis above the average over-all incidence can be attributed probably to the rather older group which develops hyperostosis frontalis interna; the majority are over 60.

In view of the known association between hepatic cirrhosis and hypertrophy of the male breast, it seemed worthwhile to consider the 16 cases of hepatic cirrhosis together. Of these 16 women 11 had fibrous and 5 had fatty breasts. In all, 7 showed proliferative change, an incidence of 43.7 percent, which is not significantly different from the over-all average.

Of the 800 women autopsied 8 died with hypothyroidism, and of these 7 showed atrophic breasts and 1 intermediate sclerosing adenosis. This high incidence of atrophy is probably significant. On the other hand, of 5 cases dying with hyperthyroidism 3 showed atrophy, 1 epitheliosis, and 1 adenosis.

Unexpectedly in 4 cases of Addison's disease, only 2 were atrophic and 1 case each showed adenosis and epitheliosis. On the other hand, of 3 cases dying with Simmonds' disease all 3 were atrophic. One case of giant-cell granuloma of the pituitary in a woman of 56 showed sclerosing adenosis (Sandison 1956). Of 5 women who died with gross obesity 3 showed atrophic breasts, 1 adenosis, and 1 epitheliosis.

Summary

There is no evidence of any breast abnormality in cases of diabetes mellitus, hyperostosis frontalis interna, or hepatic cirrhosis. Of the endocrine diseases which occurred infrequently the only possible significant finding was a high incidence of atrophy in hypothyroidism. Even here the numbers are so small that this might be chance. All 3 cases of Simmonds' disease showed atrophy also, but in Addison's disease, hyperthyroidism, and giant-cell granuloma of pituitary either adenosis or epitheliosis has been noted.

ENDOCRINE RELATIONSHIPS

In recent years enormous literature has accumulated in the field of endocrinology, so that Swyer (1956) admits that "it is most difficult, even for one professedly engaged in the speciality, to keep abreast of all the advances made." Swyer, however, makes an attempt to summarize current progress in the adrenal, sex hormone, and pituitary field. Reference may also be made to Bonser and Jull (1960) for a consideration of relationships of hormones and breast cancer. Hadfield (1956, 1958) and Hadfield and Stretton Young (1958) have reviewed recent research in physiology of the breast and in its hormonal control, with relation to mammary carcinoma. Hadfield concluded that possibly half of all mammary cancers are hormone-dependent, *i.e.*, progressive growth of such tumors is conditioned by an adequate supply of the hormones which normally govern growth of the hormone-dependent mammary-gland epithelium. Hadfield believes that normal breast growth is controlled by the synergistic action of the pituitary hormone prolactin activity at a relatively high concentration with ovarian estrogen acting at a lower concentration. Similar results may be obtained with growth hormone. For this reason oophorectomy and adrenalectomy will deprive a carcinoma of estrogenic steroids, so that if the tumor is hormone-dependent some regression of its growth may follow. However, this effect is almost invariably temporary and estrogen production is slowly re-established from some unknown site. Hadfield believes that progressive growth recurs when estrogen production reaches the level required for the promotion of normal mammogenesis. Total hypophysectomy probably deprives any tumor of estrogen and prolactin and therefore affects the growth of hormone-dependent neoplasms but has no effect on others.

Hadfield has also shown that the urine of all premenopausal and about 50 percent of postmenopausal women contains some form or forms of mammotropin. It is, in fact, thought probable that it contains prolactin. Consequently, it would seem reasonable that absence of mammotropin from the urine of a breast cancer patient would suggest that her tumor was hormone-independent and would not respond to hypophysectomy. Hadfield's work on the estimation of mammotropic potency of human urine and of the urine of postmenopausal women has been published in two papers by Hadfield and Stretton Young (1956*a* and *b*) where exact techniques, employing weanling mice, are detailed. This work seems to be very promising and further reports are awaited with interest.

Swyer (1952) enumerated the hormones of pregnancy and described assay methods. Struthers (1956) discussed postmenopausal estrogen production: In 353 postmenopausal women he found that 53 percent manifested evidence of considerable estrogenic activity. Only 20 percent showed no evidence of this. Estrogen activity apparently was not affected whether the menopause was spontaneous or surgically induced. Struthers concluded that neither ovary nor adrenal was a significant source of estrogen after the menopause, on the grounds that evidence of estrogen formation continued after oophorectomy and also after oophorectomy combined with adrenalectomy. He further concluded that estrogen was not an important factor in either breast or female genital carcinoma. This statement is based on a comparison of grading of estrogen activity between 57 women with extensive breast carcinoma, who were either postmenopausal or who had undergone opohorectomy, and a control series of women who did not have breast carcinoma but were postmenopausal or had undergone oophorectomy. The activity appeared remarkably similar in both groups. If Struthers' conclusions are valid, this corroborates Hadfield's tentative view that "prolactin" may be an important factor in hormone-dependence.

On the other hand, there can be no doubt that estrogens have a profound action on the breast. In the male, exhibition of estrogen or diethylstilbestrol produces gynecomastia, and in animals it has long been recognized that administration of estrogens produced marked changes in the breast (Burrows 1935). Willis (1960) gives an excellent summary of this aspect of experimental tumor pathology. Foote and Stewart (1945) gave an account of the effect of administration of estrogens in the adult female, with detailed descriptions of a number of cases, some of whom had been treated for menopausal symptoms and others for lumps in breast. Later specimens from biopsy or mastectomy showed a wide variety of histological appearances, which suggests that there are variable responses in individuals to estrogen administration, and that thresholds of reactivity may vary through a wide range. With one exception, the cases studied show no progressive series of changes beginning with normal breast and ending in mammary cancer. In the exceptional case marked changes were noted, but again it is doubtful whether these had any relationship to the development of the small carcinoma which was present. These changes thought to be the alleged results of estrogen therapy were florid sclerosing adenosis, papillomatosis, blunt duct adenosis, with secretory change in the cells of some lobules. Since all these phenomena may be seen, separately or together, without previous estrogen therapy, it may be unwise to assume that they necessarily result therefrom.

Emerson *et al.* (1953) published a detailed report of 18 cases of primary and recurrent carcinoma of the breast after administration of steroid hormones. They found true regression of tumor growth and cicatrization of tumor areas. Regression was preceded by necrobiosis of tumor cells and reformation of collagen followed. The degree of effect varied considerably from slight alteration to almost complete regression. They concluded that the patients with primary carcinomas characterized by marked scirrhous reaction and periductal elastosis responded best to hormone therapy. However, similar tumors failed to regress equally. It was felt that regression may be the result of an unusual hormonal stimulation of a naturally occurring process of repair. In particular the authors comment on the hyperplasia of the elastic tissue after hormone therapy. I personally have not seen gross elastosis following hormone therapy, but the few cases I have autopsied had received polytherapy. The most marked elastosis in carcinoma which I have observed followed radiotherapy.

Huseby and Thomas (1954) studied histological and histochemical alterations in the normal atrophic breast tissues of 36 patients with advanced breast cancer (more than 5 years past their menopause) who were being treated with large doses of estrogenic hormones. Of these, 34 showed evidence of epithelial stimulation, *e.g.*, enlargement of cells and nuclei with much basophilic cytoplasm in the cells; increase in the number of small ducts and formation of new lobules, some of which resembled the lobules of pregnancy, and increase in the interlobular connective tissue. There was no correlation between the degree or type of epithelial proliferation and the response of neoplasm to estrogen administration, and there was no evidence of intense panpituitary suppression after hormone administration. However, Godwin and Escher (1951) claimed that mastectomy after hormone therapy exhibited no pathognomonic change.

I refer now to the important paper of Sommers and Teloh (1952) on ovarian stromal hyperplasia in breast cancer, inspired by the "frequent association of diseases of uterus and breast, and in particular by reports of ovarian cortical stromal hyperplasia in endometrial carcinoma." At autopsy, the authors studied the ovaries from 100 cases of breast cancer. They defined cortical hyperplasia as increase in thickness and cellular density, sometimes with the formation of spherical, scalloped masses with small projecting papillary excrescences. The stromal cells are disposed in numerous whorls that weave about the capillaries. The nuclei of the stromal cells are enlarged, blunted, and contain abundant coarse chromatin granules. Hyperplastic stromal masses dip irregularly into the relatively acellular eosinophilic medullary stroma. Ovarian hilar cells were noted frequently.

Sommers and Telch found that cancer of the breast was accompanied by stromal hyperplasia in 83 percent of cases, occurring equally often in the different age groups. In a control series, hyperplasia occurred in only 37.6 percent of cases. In one case of carcinoma, hyperplasia of interstitial cells was noted. They consider that their findings are histological evidence for excess of estrogen secretion in cases of breast carcinoma.

Burt (1954) investigated the pituitary in 19 cases of postmenopausal women with various degrees of ovarian stromal hyperplasia and listed many references on this particular subject. Her study demonstrated that in the 5 patients with the most severe stromal hyperplasia the anterior lobe of the pituitary showed increase in "hypertrophic amphophils" but no significant differences in other cell types. The "hypertrophic amphophil" cell is an agranular cell whose most striking characteristic is a giant nucleus, often of irregular outline. The cytoplasm is scanty, may be foamy, and the cell outline may be indistinct and irregular. These cells may be derived from "amphophils" which contain Schiff-positive granules less numerous than those in normal beta cells and stain less intensely with Schiff reagent. The nucleus is often large and irregular. They also possess some staining property of the acidophils and "amphophils" is therefore suggested as an accurate designation. Burt suggests that the amphophil series may produce trophic hormones stimulating the gonads, thyroid, and adrenals. This point is of interest in view of my finding that of 8 women with hypothyroidism, 7 showed atrophic breasts; of 4 such cases studied the ovarian stromal cellularity was designated only as + + in 3 and the ovaries generally as fibrotic in the 4th. The pituitaries in these cases showed thyroprival cells.

Burt and Castleman (1953) in a previous paper showed that in postmenopausal women with carcinoma of the breast, the normal beta cells and "hypertrophic amphophils" of the pars anterior were significantly increased compared with women who had no history of breast carcinoma. They state that this abnormality is not pathognomonic of breast cancer but suggests some type of adrenal overactivity. Estrogen therapy produces a decrease in the levels of "hypertrophic amphophils" both in women who have undergone oophorectomy and those who have not. If therapy is continued and oophorectomy is not done, there is a rise in the normally granulated beta cells, but if oophorectomy has been done, estrogen therapy produces a decrease in beta cells and a rise in the proportion of alpha cells. They discovered no evidence that estrogen administration led to the formation of chromophobe adenomas in these patients. Finally, there was no difference in the response in women who had regression of their breast carcinoma and those who did not, as a result of the estrogen therapy.

In an interesting paper, Sommers (1955) discusses endocrine abnormalities in 207 women with breast carcinoma and 248 noncancerous controls. He noted ovarian stromal hyperplasia in 86 percent of his cancer series and 37 percent of controls; endometrial hyperplasia in 67 percent and 27 percent of controls; duct hyperplasia of the remaining or uninvolved breast in 44 percent—no controls were available; amphophil hyperplasia of the pituitary in 36 percent and 12 percent of controls; and pancreatic islet hyperplasia in 21 percent and 5 percent of controls. There was less striking statistical evidence of hyperplasia of the zona fasciculata and reticularis of the adrenal, ovarian hilus cells, and pituitary alpha cells in the cancer series compared with the controls. On the other hand, Sommers indicated that 59 percent of his cancer series had thyroid atrophy compared with 22 percent of controls and adenomatous goiter in 13 percent compared with 6 percent of controls.

In my small series of carcinoma cases I have not confirmed Sommers' findings of great predominance of hyperplasias compared with control series, but this may be due to the therapeutic measures adopted before death, which are designed to alter endocrine balance considerably.

Results

In this study of 275 cases, no elaborate or time-consuming investigations such as differential cell counts of the pituitary could be carried out. The following findings are those obtainable on simple histological investigations only: Of the 275 cases, 241 were graded for ovarian stromal cellularity by the use of an arbitrary + + +, + +, + method; + + + being highly cellular, + + moderately cellular, and + poorly cellular. Note was also taken of: simple cysts; the presence of prominent interstitial cells at the hilum; the presence of tubules at the hilum representing vestigial elements, etc. Of the 34 cases not graded, 15 had certainly had surgical oophorectomy; 4 had advanced neoplasm replacing the ovaries; in a few instances the ovaries were exceedingly atrophic, tiny, or calcific, and in the remainder could not be identified with certainty because of pelvic infection, adhesions, or neoplasm. In a few of these cases it is possible that oophorectomy had been carried out (figs. 43 through 48).

Some of the incidental interesting findings were: 2 small invasive granulosa-cell tumors; 2 Brenner's tumors, a fibroma of the ovary; 2 metastatic carcinomas of stomach, 1 of bronchus, and 1 of corpus uteri; and infiltration by leukemia, Hodgkin's disease, and medullary reticulosis tissue. Other conditions included endometriosis, polyarteritis of the ovarian hilum, and several instances of foreign-body reaction of giant-cell type (figs. 49 through 52).

Oophorectomy had been done for advanced breast carcinoma in 4 cases, for gynecological or other reasons in 11. Of these 11 women, 4 had atrophic breast parenchyma but 2 showed adenosis: 1 showed sclerosing adenosis: 4 showed epitheliosis and these included 1 with adenosis also. Advanced neoplasm replacing ovary occurred in 4 women. Of these 3 showed atrophic breasts and 1 showed adenosis.

Of the 241 cases in which stromal cellularity was graded, 147 were + + +, 75 + +, and only 19 +. In general, there was less stromal cellularity in the older age groups, *i.e.*, when cellularity was + 91 percent of all cases were over 50, + + 80 percent were over 50, and + + + 71 percent were over 50. Only after 70 did + + + cellular stroma become less frequent than + + and + combined. The maximum incidence for + cellularity was from 76 to 80 years. Even in the period 86 to 90, one of the 2 cases was + + and the other + + + cellular (table 32).

The age range of the 147 cases with stromal cellularity of + + + was 21 to 90. Of these, 6 were "normal" breasts showing no features of interest (4.1%) and 70 were atrophic, 47.6 percent, but of these 70 cases, 58 were in women over 50 (39.5%) and, in these women, atrophy might be regarded as "normal" for this age group. In all, 51 cases showed some

Table 32.—Ovaries—stromal cellularity*

	+		+ +		+ + +		
Age group	Number	Percent	Number	Percent	Number	Percent	Total
21–25	—		1	20. 0	4	80. 0	5
26–30	—		—		5	100	5
31–35	—		2	15. 4	11	84. 6	13
36–40	—		4	40. 0	6	60. 0	10
41–45	1	7. 1	3	21. 3	10	71. 6	14
46–50	—		5	41. 7	7	58. 3	12
51–55	2	5. 7	6	17. 1	27	77. 2	35
56–60	2	7. 4	8	29. 6	17	73. 0	27
61–65	3	7. 1	15	35. 3	22	57. 6	40
66–70	2	5. 3	13	34. 2	23	60. 5	38
71–75	3	12. 5	11	45. 8	10	41. 7	24
76–80	5	41. 7	4	33. 3	3	25. 0	12
81–85	1	25. 0	2	50. 0	1	25. 0	4
86–90	—		1	50. 0	1	50. 0	2
21–90	19	7. 9	75	31. 1	147	61. 0	241

*+ = 91 percent of cases aged over 50; + + = 80 percent of cases aged over 50; + + + = 71 percent of cases aged over 50.

form of adenosis, 34.7 percent, but of these 22 occurred under age 50 when adenosis might be regarded as "normal," *i.e.*, 14.9 percent. If we combine these variants of normal our incidence becomes 58.5 percent with "significant proliferative" lesions (*i.e.*, excluding adenosis under age 50) representing 33.3 percent. Of the 147 cases, 36 women, 24.5 percent, showed significant epithelial hyperplastic change, *i.e.*, epitheliosis, papillomatosis, or carcinoma (table 33).

The 75 cases in which stromal cellularity was + + ranged in age from 21 to 90. Only 2 breasts (2.7%) showed no feature of interest, but 40 cases (53.3%) were atrophic. However, of these 40 cases, 38 occurred

Table 33.—Breast with ovarian stroma + + + cellularity

Age group	Total No. of cases	Normal	Atrophic	All cases with adenosis and/or sclerosing adenosis	All cases with epitheliosis, papillomatosis, or carcinoma	All cases with proliferative change
21–25	4	1	1*	2	—	2
26–30	5	—	1	4	1	4
31–35	11	4	2	4	2	5
36–40	6	1	—	5	3	5
41–45	10	—	3	5	3	7
46–50	7	—	5	2	—	2
51–55	27	—	17	9	2	10
56–60	17	—	9	5	6	8
61–65	22	—	11	6	7	11
66–70	23	—	13	5	7	10
71–75	10	—	4	4	4	6
76–80	3	—	3	—	—	—
81–85	1	—	—	—	1	1
86–90	1	—	1	—	—	—
21–90	147	6(4. 1%)	70(47. 6%)	51(34. 7%)	36(24. 5%)	71(48. 3%)

*Failure to develop lobules.

after age 50 when atrophy might be regarded as the normal state, *i.e.*, 50.7 percent. In all, 22 cases showed some form of adenosis, but of these 10 occurred under 50 when adenosis might be regarded as a normal variant, 13.3 percent. If we combine these normal variants our incidence becomes 66.7 percent, with "significant proliferative" lesions (excluding adenosis under age 50) as 31.6 percent. Of the 75 women concerned, 16 or 21.3 percent showed significant epithelial hyperplastic change, *i. e.*, epitheliosis, papillomatosis, or carcinoma (table 34).

TABLE 34.—Breast with ovarian stroma + + cellularity

Age group	Total No. of cases	Normal	Atrophic	All cases with adenosis or sclerosing adenosis	All cases with epitheliosis, papillomatosis, or carcinoma	All cases with proliferative change
21–25	1	1	—	—	—	—
26–30	—	—	—	—	—	—
31–35	3	—	—	3	—	3
36–40	3	1	—	2	—	2
41–45	3	—	—	3	—	3
46–50	5	—	2	2	1	3
51–55	6	—	3	3	1	3
56–60	8	—	5	2	1	3
61–65	15	—	8	3	5	7
66–70	13	—	7	3	6	6
71–75	11	—	10	—	1	1
76–80	4	—	3	1	—	1
81–85	2	—	1	—	1	1
86–90	1	—	1	—	—	—
21–90	75	2(2. 7%)	40(53. 3%)	22(29. 3%)	16(21. 3%)	33(44. 0%)

Finally, we must consider the 19 cases with stromal cellularity of + only, the age range here being restricted from 41 to 85. In this group there were no normal breasts with no features of interest. Ten cases, however, were atrophic, 52.6 percent, and 9 of these occurred after age 50 when atrophy might be regarded as the normal state, 47.7 percent. Only 4 (21.1%) showed adenosis and these were all over 50. Epithelial proliferative change, *i.e.*, epitheliosis, papillomatosis, or carcinoma, occurred in 7, 36.8 percent (table 35).

If we tabulate our results thus:

	All adenosis	Epithelial proliferation	All proliferative change	"Normal"	Significant proliferation
	Percent	Percent	Percent	Percent	Percent
+ + +	34. 7	24. 5	48. 3	58. 6	33. 3
+ +	29. 3	21. 3	44. 0	66. 7	31. 6
+	21. 1	36. 8	47. 4	47. 7	47. 4

We see that the breast in women with + + + cellular ovarian stroma is associated with a higher incidence of adenosis, epithelial proliferation, all proliferative change, and significant proliferative lesions than those with + + cellular stroma. Where the ovarian stroma is only + cellular, though the incidence of adenosis is lower, epithelial proliferation and

TABLE 35.—Breast with ovarian stroma + cellularity

Age group	Total No. of cases	Normal	Atrophic	All cases with adenosis or sclerosing adenosis	All cases with epitheliosis, papillomatosis, or carcinoma	All cases with proliferative change
21–25	—	—	—	—	—	—
26–30	—	—	—	—	—	—
31–35	—	—	—	—	—	—
36–40	—	—	—	—	—	—
41–45	1	—	1	—	—	—
46–50	—	—	—	—	—	—
51–55	2	—	1	1	1	1
56–60	2	—	—	1	1	2
61–65	3	—	3	—	—	—
66–70	2	—	1	—	1	1
71–75	3	—	1	—	2	2
76–80	4	—	3	—	1	1
81–85	2	—	—	2	1	2
86–90	—	—	—	—	—	—
21–90	19	—	10(52. 6%)	4(21. 1%)	7(36. 8%)	9(47. 4%)

significant proliferative lesions are higher than in either + + + or + + cellular stroma cases. All proliferative lesions are, however, a little less frequent than in + + + cellular stroma cases. This discrepancy is explicable on the assumption that cases with only + cellular stroma are usually older than those in the other two groups and that, as we have seen previously, epitheliosis is more frequent in older women. This does not vitiate our comparison between cases with + + + and + + stroma, because in these the cases with + + are less frequent proportionately in the younger age groups and more frequent proportionately in the older groups. With regard to gross breast types the fibrous is most common in the + stroma and least common in the + + . This probably reflects the older age group in + stroma (atrophic breasts) and the higher proportion of virginal fibrous breasts in + + + than + + stromas.

I have made similar analyses based on other findings in the ovary. If we consider these cases in which the ovaries showed continuing luteal or follicular activity, we find that these number 31 in all and are in the age group 21 to 55. This group shows a low incidence of atrophy (16.1%), as might be expected, and a relatively high proportion of proliferative change (67.8%). Both normal (*i.e.*, unexceptional) breast parenchymas (16.1%) and all forms of adenosis (64.5%) are more frequent than the over-all incidence for this age group. Epithelial proliferative change (12.9%) is only a little higher than the over-all incidence for this age group (10.9%), but the difference is probably not significant.

When ovarian cysts (other than follicular cysts) are present in the ovaries, the incidence of adenosis (30.4%) is similar to the over-all incidence of 31.9 percent, and epithelial proliferation (21.4%) is only slightly less than the over-all incidence of 24.5 percent. When residual tubules at the hilum persist, the incidence of adenosis is 32.8 percent (over-all incidence 31.9%) and epithelial proliferation is 29.3 percent (slightly higher than the over-all incidence of 24.5%). When we view the 18 cases in which interstitial cells at the hilum are prominent, the incidence of 55.5 percent adenosis is significantly high but epithelial proliferation at 22.2 percent is lower than the over-all incidence of 24.5 percent.

The pituitary was examined routinely in 275 cases. Few significant results emerge with regard to relationship between pituitary and breast appearance. However, before examining these we may note the incidence of some phenomena in the pituitary in this moderately large series of cases. Beta cells were present in the pars nervosa in about 33 percent and although present at all ages were most commonly seen in middle and late life. Lipofuscin pigment in pars nervosa was present in about 20 percent of all cases, and was most frequently seen in the seventh decade. Some relative increase in beta-cell prominence was noted in about 15 percent and was again most frequently seen in older women. Alpha-cell prominence was rare (about 5%), probably because the alpha cells are normally the most frequent cells in the pars anterior and small increases are likely to escape detection unless detailed counts are done. Relative chromophobe prominence was a little more common (about 10%) and occurred at all ages. Hyalinization of thickened stroma in some areas of pars anterior occurred in about 17 percent and was more frequent in older women. Chromophobes of hyperchromatic, small fetal type occurred in about 12 percent of cases, especially in the middle period of life. Some vacuolation of beta cells occurred in about 45 percent and was more frequent with increasing age. Ciliated small cysts of Rathke type occurred in about 5 percent of cases, simple colloid intermediate cysts of appreciable size in about 4 percent, and small cysts in the substance of pars anterior in about 1.5 percent. Three cases showed small infarcts of pars anterior and squamous rests were noted in 7. Granular cells of Priesl-Sternberg type were noted in 5 women. In 3 there appeared to be some hyalinization of the alpha cells. In all, of 275 cases 19 showed simple adenoma, of which 13 were of chromophobe, 5 of beta-cell (none showing Cushing's syndrome), and 1 of alpha-cell type (without acromegaly). In some cases already described in the section on mammary carcinoma, some necrosis had been produced in the pituitary by transnasal introduction of radioactive material (figs. 53 through 59, and tables 36 through 39).

With regard to the pituitary in endocrine disorders, the presence of thyroprival cells in hypothyroidism, of diminution of beta cells in Addison's disease, and of atrophy and fibrosis in Simmonds' disease have all been confirmed. I have described the case of giant-cell granuloma of the pituitary elsewhere (1956). The pituitary in obesity has not shown a consistent picture, and in 1 case a tiny basophil adenoma was noted, of the type sometimes seen in Cushing's syndrome. Obesity is almost certainly the result of a prepituitary lesion.

As to the appearance in the breast associated with some of these phenomena, we find that few significant relationships emerge. In these women with vacuolation of beta cells proliferative lesions are rather less

TABLE 36.—Breast with pituitary showing vacuolation of basophils

Age group	Total No. of cases	Normal		Atrophic		Cases showing adenosis and/or sclerosing adenosis	Cases showing epitheliosis, papillomatosis, or carcinoma	All cases with proliferative lesions	
		Number	Percent	Number	Percent			Number	Percent
21–25	2	1	50	—		1	—	1	50
26–30	1	—		1	100	—	—	—	
31–35	4	3	75	—		1	1	1	25
36–40	7	2	28. 6	—		3	2	5	71. 4
41–45	5	—		1	20	2	3	4	80
46–50	5	—		2	40	3	—	3	60
51–55	16	—		9	56. 3	6	—	7	43. 7
56–60	10	—		7	70	3	2	3	30
61–65	21	—		11	52. 4	7	6	10	47. 6
66–70	20	—		14	70	2	5	6	30
71–75	18	—		12	66. 7	1	5	6	33. 3
76–80	8	—		6	75	1	1	2	25
81–85	5	—		1	20	3	3	4	80
86–90	2	—		2	100	—	—	—	
21–90	124	6	4. 8	66	53. 2	33 (26. 6%)	28 (22. 6%)	52	42. 0

TABLE 37.—Breast with pituitary basophils prominent

Age group	Total No. of cases	Atrophic		All cases showing adenosis and sclerosing adenosis	All cases showing epitheliosis or carcinoma	All cases showing proliferative lesions	
		Number	Percent			Number	Percent
21–25	—	—		—	—	—	
26–30	1	1		—	—	—	
31–35	—	—		—	—	—	
36–40	1	—		1	—	1	100
41–45	2	—		2	—	2	100
46–50	2	—		2	—	2	100
51–55	4	2	50	2	—	2	50
56–60	1	—		—	1	1	100
61–65	8	4	50	—	4	4	50
66–70	7	5	71. 4	1	2	2	28. 6
71–75	9	6	66. 7	1	3	3	33. 3
76–80	2	1	50	—	1	1	50
81–85	2	—		2	—	2	100
86–90	—	—		—	—	—	—
21–90	39	19	48. 7	11(28. 2%)	11(28. 2%)	20	52. 3

common than normal, especially epithelial hyperplasia. Cases showing beta cells in pars nervosa, lipofuscin in pars nervosa, and those with some prominence of basophils show roughly similar figures for atrophy and proliferation. On the other hand, those 15 cases showing eosinophils more dominant than usual revealed a low incidence of adenosis but a normal incidence of epitheliosis, while those showing some prominence of chromophobes had a rather high incidence of adenosis and a low incidence of epitheliosis. Both of these phenomena occurred in about the same age distribution so that age cannot be invoked to explain the difference in the breast histology. Stromal hyalinization, occurring in the elderly group, showed a higher incidence of both types of proliferative change despite a

TABLE 38.—Condition of breast with pituitary eosinophils prominent

Age group	All cases	Atrophic		All adenosis/sclerosing adenosis		All epitheliosis and carcinoma		All proliferative lesions	
		Number	Percent	Number	Percent	Number	Percent	Number	Percent
21–25	—	—		—		—		—	
26–30	—	—		—		—		—	
31–35	—	—		—		—		—	
36–40	1	—		—		1	100	1	100
41–45	3	1	33. 3	1	33. 3	1	33. 3	2	66. 7
46–50	1	1	100	—		—		—	
51–55	1	1	100	—		—		—	
56–60	1	1	100	—		—		—	
61–65	4	2	50	1	25	1	25	2	50
66–70	2	1	50	—		1	50	1	50
71–75	1	1	100	—		—		—	
76–80	—	—		—		—		—	
81–85	1	—		—		1		1	
86–90	—	—		—		—		—	
21–90	15	8	53. 3	2	13. 3	5	33. 3	7	46. 7

TABLE 39.—Condition of breast with pituitary chromophobes prominent

Age group	All cases	Atrophic		All adenosis/ sclerosing adenosis		All epitheliosis and carcinoma		All proliferative lesions	
		Number	Percent	Number	Percent	Number	Percent	Number	Percent
21–25	—	—		—		—			
26–30	2	—		2	100	—		2	100
31–35	2	1	50	1	50*	—		1	50
36–40	1	—		1	100	—		1	100
41–45	2	—		2	100	—		2	100
46–50	2	2	100	—		—		—	
51–55	6	4	66.7	2	33.3	1	16.7	2	33.3
56–60	3	—		2	66.7	2	66.7	3	100
61–65	4	3	75	1	25	1	25	1	25
66–70	1	—		—		1	100	1	100
71–75	3	3	100	—		—		—	
76–80	1	1	100	—		—		—	
81–85	—	—		—		—		—	
86–90	—	—		—		—		—	
21–90	27	14	51.9	11	40.7	5	18.5	13	48.1

* Adenosis of pregnancy.

presumed decrease in the number of active pituitary cells. The presence of chromophobe cells of fetal type appeared to be associated with a rather low incidence of proliferative change, especially of adenosis. No significant trend was noted in the small number of cases showing Rathke or simple intermediate cysts, cysts of pars anterior, recent infarction of pars anterior, squamous rests, or granular cells of Priesl-Sternberg type. Three cases with hyalinization of alpha cells showed atrophic breast parenchyma, but this may be simply a coincidence.

Finally it may be said that in considering the various major proliferative changes in the breast in previous sections it was not possible to demonstrate any consistent pituitary picture.

Summary

Although in this study some interesting figures of the frequency of certain phenomena in the pituitary and ovary emerged, little or no information of value concerning breast-ovary-pituitary relationship has been obtained. It seems likely that this problem is one calling for a biochemical or biological assay approach, possibly along the lines of Hadfield and Stretton Young (1956), rather than a histological one.

However, certain simple statements may be made, *e.g.*, that oophorectomy is not always followed by atrophy of the breast parenchyma, and that the breast of the castrated woman may show adenosis or epitheliosis. Most ovaries have a quite cellular stroma even after the menopause, although a low stromal cellularity tends to be confined to the older woman. If stromal cellularity is divided into three grades, the highest grade is associated with a higher incidence in the breast of proliferative change, both lobular and epithelial, than the moderate one. Nevertheless, the incidence of epithelial hyperplasia is highest in the lowest-grade stroma. The numbers involved are small, but if the result is significant it may be explained by the higher age of women comprising this group. It may be definitely stated in any case that a low-grade ovarian stromal cellularity may be still accompanied by any form of proliferative breast lesions including carcinoma.

Not unnaturally, the presence of follicular or luteal activity is accompanied by a low incidence of atrophy and a high rate of adenosis, but this might readily be predicted on the age distribution of these phenomena and their association with hormonal cyclical activity. The only other apparently significant relationship in the ovary is the association of a high incidence of adenosis with prominent interstitial cells at the hilum.

With the pituitary we are on even less secure ground. All that may be said is that beta-cell vacuolation appears to be associated with a lower rate of proliferative change; that alpha-cell prominence appears to be associated with a low incidence of lobular adenosis, and that prominence of chromophobes is associated with a rather high incidence of lobular adenosis. In my small series of carcinoma cases, the number of instances in which the pituitary was unaffected by hormone or radioactive implant was even smaller, but I did not find any significant increase in beta-cell activity.

THE MALE BREAST

In the neonatal period and during childhood the male breast is similar to and grows in the same way as the female breast. At adolescence, however, the female breast develops more or less rapidly with growth of both ducts and periductal stroma, though full lobular development is not usually seen until ovulation is established. Jung and Shafton (1938) believed that the male adolescent breast undergoes a slight degree of enlargement which later partially regresses. At this period estrogen and androgen are in the urine in unusual amount.

The normal adult male breast is rarely prominent except when some degree of hypertrophy is present. In some men fat accumulates under the skin in the pectoral region and may produce an appearance of pseudogynecomastia, which is almost invariably bilateral.

In the present series of 500 cases (for age distribution *see* table 40) 21 men or 4.25 percent had fibrous nodes of breast tissue which were larger than normal and yet did not show the appearance of true hypertrophy. In the remaining cases the breast was represented by a small node of fibrous tissue in which occasional simple ducts were present or in more obese males by a small fibrofatty node in which simple ducts could again be discerned. The stroma was dense and collagenous and there was no periductal fibrillary tissue (figs. 60 and 61).

The ducts showed a simple epithelial lining, often two cells thick and of cuboidal appearance. Dilatation of ducts or "ectasia" was present in

30 of 500 cases, in 6 percent. Duct ectasia was relatively more frequent in the younger age groups. Quite frequently the duct epithelial cells showed small globular projections into the lumen of the ducts and scanty eosinophilic material was sometimes seen. Around the ducts myoepithelium was often discerned when stained by the phosphotungstic acid-hematoxylin or Lendrum's method (1951), especially if the sections were cut rather tangentially. In older men the ducts were sometimes rather atrophic and collapsed so that they became flat and linear in appearance on longitudinal section. Pacinian tactile corpuscles were seen in 103 of 500 cases, *i.e.*, in 20.6 percent, whereas in the female they were noted only infrequently, in 2.4 percent of 800 cases.

TABLE 40.—Male breasts—all cases

Age group	Number of cases
11–15	1
16–20	6
21–25	6
26–30	11
31–35	6
36–40	18
41–45	25
46–50	45
51–55	54
56–60	58
61–65	83
66–70	82
71–75	54
76–80	34
81–90	11
91–100	1
Age uncertain	5
Total	500

Subareolar apocrine glands were observed in 261 of 500 cases, an incidence of 52.2 percent, but apocrine metaplasia was noted in the ducts in only 3 or in 0.6 percent, while in the female the corresponding figure was 39.2 percent (figs. 62 and 63).

Thickening of the periductal basement membrane was seen in 36 of 500 cases, an incidence of 7.2 percent. This is a phenomenon of aging and was seen most frequently from 66 to 90 years.

Periductal lymphocytes were noted in 24 out of 500 cases, an incidence of 4.8 percent. They were most frequently seen in the period 56 to 70 years. Lymphocytes were noted in the female breast in 21.0 percent of over-all cases.

Mammalithiasis was noted in only 1 male (age 69), an incidence of 0.2 percent. This compares with an incidence in the female of 0.5 percent; mammalithiasis in both sexes appears to be a rare condition.

In the present series 1 case of hemosiderosis was noted in the male breast as well as 1 in the female. In the female, hemosiderin was present in epithelium and to a lesser extent in the myoepithelium of the ducts and in the acinar epithelium. In the male, the hemosiderin was present in the stroma and only in very small amount in the duct epithelium.

It may be said that in the breast of 36 coal miners examined at autopsy there was no evidence of anthracosis. In 1 case there was dermatitis of the areolar skin and in another the cutis under the areola contained a small implantation cyst with giant-cell reaction. The cutis contained round-cell infiltrates in 3, and there was a lymphocytic infiltration about the sweat glands in 8. In one 65-year-old coal miner the breast tissue proper, on one side, contained a small giant-cell granuloma which did not contain any doubly refractile material; the pituitary was normal and it seems probable that this lesion represents a reaction to a ruptured duct.

GYNECOMASTIA AND EPITHELIOSIS

Karsner (1946) aptly summarized the essential features of hypertrophy of the male breast as follows, "Gynecomastia is an enlargement of the mammary gland or glands of males due to proliferation of connective tissue, dense in the general stroma, and often loosely arranged in periductal regions, together with variable degrees of multiplication, elongation or branching of ducts, or of all three, without formation of true acini, accompanied by periductal or more widespread infiltration of lymphocytes, plasma cells, large mononuclear cells, and occasionally eosinophiles or neutrophilic polymorphonuclear cells, or both: secretion is frequently present in ducts, may be discharged spontaneously or manually expressed, but rarely if ever is it true colostrum or milk. This definition excludes pseudogynecomastia, due to the deposition of fat in the mammary regions, as well as suppurative or other essentially inflammatory processes, granulomatous lesions, and neoplasma, either benign or malignant." This comprehensive definition introduces the various features which may be present in diverse combinations in different cases of gynecomastia.

Karsner's paper is based on a series of 280 cases, whose ages varied from 14 to 77 years with a maximal incidence in the decade 21 to 30. Both breasts were affected in 12 cases, the right only in 138 and the left only in 124. The duration of the condition varied from 3 months to many years. Karsner discussed the histological changes in the stroma and epithelium. He points out that mitotic activity may be noted in the latter not infrequently and there may be considerable hyperplasia of epithelium, sometimes papillary in pattern. True intraductal papilloma is, however, rare.

Karsner believes that formation of true acini never occurs. However, I have seen apparent acinar formation in the male breast after administration of diethylstilbestrol for a period of 9 years for what was considered to be carcinoma of prostate (fig. 67). The only other male breasts which I have seen with well-formed lobules have been cases of chromatin-positive Klinefelter's syndrome. Duct distension occurred in 63 of Karsner's cases, *i.e.*, in 22.5 percent, and squamous metaplasia was seen in 3. No case of supervening neoplasm was observed. In postmortem specimens apocrine glands were seen under the areola in 37 cases.

Karsner concluded that atrophy of the testis could not be significant in the development of gynecomastia but thought that influence of the interstitial cells was not clear and that the hypophysis might have some role. For a brief account of breast enlargement in adolescence, reference may be made to Jung and Shafton (1938) who point out that in many boys at the age of 13 a subareolar node begins to appear, enlarges for just over a year, and then slowly declines to half its maximal size at 17 years.

Much has been written on the effect of hepatic cirrhosis on breast, testis, and prostate of men. A good reference is the paper of Bennett, Baggenstoss, and Butt (1950). There seems no doubt that in a proportion of such cases there is gynecomastia, testicular atrophy, and benign hypertrophy of the prostate. There seems to be a distinct relationship between hypertrophy of the male breast and urinary excretion of estrogens. In men with hepatic cirrhosis it seems reasonable to assume that the cirrhotic liver is incapable of adequately detoxicating estrogens which are normally rapidly destroyed in the male. I have shown that active budding of mammary ducts occurred in a 7-year-old girl who died of hepatitis and cirrhosis (1956). There is no doubt that hypertrophy of the male breast may accompany certain neoplasms of the testis, *e.g.*, chorionepithelioma in teratoma, interstitial-cell tumors, and some adrenal tumors (Willis 1952). It is possible that in a few cases of testicular atrophy there may be a decline in androgen production which might explain senile gynecomastia, without evidence of endocrine tumor or liver disturbance. As mentioned, diethylstilbestrol may produce hypertrophy of the male breast and has been noticed not only in cases of therapeutic administration of diethylstilbestrol but also in diethylstilbestrol workers (Scarff and Smith 1942). For an interesting report on myoepithelium in gynecomastia, reference may be made to Karnauchow (1954) who claims that in the majority of cases the myoepithelial element is prominent.

Kuzma (1953) claims that gynecomastia is about 3 times more common than cancer of the male breast. In my surgical series of breast cases collected over a 5-year period in this department there were 19 specimens from males and 1,010 from females (1958). Of the 19 male cases, 1 was inflammatory, 3 were carcinomatous, and 15 were hypertrophic, a ratio of 5:1 in favor of hypertrophy.

Results

Of the 500 males examined, 15 or 3 percent showed the typical appearance of hypertrophy, both stromal and epithelial; 4 were unilateral and the remainder bilateral, though the degree of involvement was not necessarily equal. In 3 there was an unusual degree of epithelial proliferation which I classify as "epitheliosis" of the male breast. Epithelial hyperplasia or epitheliosis without hypertrophy of the stromal element occurred in 8 of the 500 cases, an incidence of 1.6 percent. This was usually bilateral and most frequently of papillary type, though a cribriform pattern has been noted and was sometimes rather clear-celled. In none of the cases was there any great evidence of atypical cytology, in the sense of precancerous change (figs. 64 through 69).

The ages of these cases varied from 16 to 84 years. When there was hypertrophy without marked epithelial proliferation the maximum number of cases occurred in the seventh decade, but considered as percentages of all cases in these periods the maximum relative incidence is in the young, *i.e.*, 1 case in 7 between 11 to 20, 1 in 17 between 21 to 30, and in the very old, *e.g.*, 1 in 11 between 81 and 90 years. Concerning epitheliosis, we find however that the absolute maximal incidence is in the sixth decade and the relative incidence in this decade is the same as in the second and eighth although here only 1 and 2 cases, respectively, are concerned.

The primary cause of death in these cases was: Three with hypertrophy had gross carcinomatosis of the liver; 2, hepatic cirrhosis (one of these was Kinnier Wilson's disease in a boy aged 16); 2, hypertension and mild fatty change in the liver; 1 died from perforated duodenal ulcer; 1, intestinal obstruction in a man with hyperostosis frontalis interna; 3 with hypertrophy had mitral stenosis with chronic venous congestion of the liver, 2 of these showed epitheliosis; of 3 receiving diethylstilbestrol therapeutically, 1 for malignant melanoma and 2 for clinical carcinoma of prostate, 1 of these at autopsy proved not to have had carcinoma of the prostate but undoubtedly the other had clear-cell carcinoma and 1 had epitheliosis. Most of these cases showed testicular atrophy with thickening of the peritubular basement membranes.

In epitheliosis without hypertrophy of the breast generally we find that 1 man had calcific aortic stenosis with chronic venous congestion of liver; 1, dissecting aortic aneurysm with fatty liver; 1, mitral stenosis without gross venous congestion; 1, a fractured skull; 1, renal carcinoma; 1, bronchial carcinoma; 2, chronic duodenal ulcers; and 2, myocardial infarction.

If we now consider these 23 cases together we see that only 2 had hepatic cirrhosis, but 3 had hepatic carcinomatosis; 4 had marked chronic venous congestion of liver and 3 fatty livers; 11 had no evidence of hepatic change of appreciable degree. Of these 3 had duodenal ulcers and 2 coronary artery disease. It is worth mention that in the series of 500 cases studied, 1 with Kinnier Wilson's disease showed gynecomastia; only 1 of 8 cases of multilobular cirrhosis showed gynecomastia; of 3 cases of postnecrotic scarring of the liver none showed gynecomastia nor did 1 with hemochromatosis.

Papillomatosis was noted in 5 cases whose ages ranged from 40 to 73 years. In no instance was there true papilloma but in these 5 there was papillary proliferation into the ducts with central fibrovascular cores present. In 1 of these there was chronic venous congestion of the liver; in another mild fatty change. One had chronic duodenal ulcer.

Summary

Of 500 cases examined, 23 or 4.75 percent showed either hypertrophy of the breast, epitheliosis of the breast, or both conditions. The relative maximal incidence of gynecomastia was in the young or very old. Among the most common apparent causes in this small series were diethylstilbestrol therapy, hepatic carcinomatosis, chronic venous congestion of the liver, and multilobular cirrhosis. Nevertheless, in this series multilobular cirrhosis was only infrequently accompanied by gynecomastia. No case of testicular or adrenal tumor was noted.

Papillomatosis, but not true papilloma, was also noted in 5 cases.

NEOPLASIA IN THE MALE BREAST

Neoplasms of the male mammary gland are rare. In my series of surgical cases, I examined 1,010 female and 19 male specimens. Of these 19 specimens 1 was inflammatory, 15 hypertrophic (gynecomastia), and only 3 were carcinomatous. These latter occurred in the sixth, seventh, and ninth decades. The oldest man, aged 81, had a long history of local excision, radiotherapy, and diethylstilbestrol therapy. The tumor was an infiltrative spheroidal cell carcinoma with some mucoid change. The second, aged 61, had a history of lump in the breast for 1 year, and showed both intraductal and infiltrative scirrhous carcinoma and nodal metastasis. The third, a man aged 53, had been ill for 4 months only and showed a rather clear-celled intraductal and infiltrative carcinoma with metastasis to lymph nodes; he also showed mammalithiasis.

For a valuable review of carcinoma of the male breast with a report on 146 cases, reference may be made to Treves and Holleb (1955) who state that cancer of the male breast accounts for less than 1 percent of breast cancer in both sexes. (In my surgical series, 3 male breast cancers of a total of 527 cases represented an incidence of about 0.5% only.) The age range of their series was 24 to 85 years. The left breast was involved more frequently than the right. Bilateral neoplasia was present in 2.7 percent of all cases. The majority of cases was infiltrating duct carcinoma, but all types except lobular carcinoma were noted. Treves (1949) had previously recommended castration in cases of inoperable breast carcinoma in males, but Treves and Holleb do not recommend this as a substitute for mastectomy in operable cases. Other valuable papers on carcinoma of the male breast are those of Sachs (1941) and Gilbert (1933). For reviews of Paget's disease of the male breast, reference may be made to Treves (1954) and Sandison (1956).

Cheatle and Cutler (1931) devote a chapter to tumors of the male breast and discuss the different types which have been described. They state that of all breast tumors in the male, 87.5 percent are malignant (carcinoma 85.5% and sarcoma 2%) and 12.5 percent are benign (fibroadenoma 10%, papilloma 1.5%, and rare tumors, such as fibroma, myxoma, lipoma, chondroma, etc., 1%). Cheatle and Cutler's description of fibroadenoma rather suggests that under this name they may include some instances of gynecomastia.

Geschickter (1945) also devotes a chapter to tumors of the male breast. In his series of 150 cases there were 108 cases of gynecomastia, 30 carcinomas, 3 sarcomas, 2 lipomas, and 1 papilloma.

Geschickter equates fibroadenomatous hypertrophy with gynecomastia but appreciates that this is not a truly neoplastic condition.

Results

In the present series of 500 cases, neoplasm was rare. One interesting case was that of a 66-year-old man admitted to the hospital with a pathological fracture of femur, which proved to be the result of bone metastasis from a carcinoma of the left breast. At autopsy the breast had an infiltrative carcinoma arising on an intraductal basis with Paget's disease of the nipple. Metastasis to the pars nervosa of the pituitary had occurred. This case has been fully described elsewhere (1956) (figs. 71, 72, and 73).

There was no other case of primary carcinoma although 1 man aged 47 showed secondary melanoma from a primary site on the eyebrow. He had been treated (to no avail) with diethylstilbestrol and this produced some hypertrophy of his breasts. Another 71-year-old man showed bilateral lymphatic permeation of the breasts from a primary carcinoma of bronchus.

In 1 man aged 78 there was a small area, 4 × 3 mm, of apparent fibroadenoma in one breast. In this area the stroma was of rather clear fibrillary type and contained, here and there, a mild infiltrate of lymphocytes, mononuclears, and mast cells. The pattern was pericanalicular except in one space where there was apparent intracanalicular polypoid projection. The lining epithelium was a little hyperplastic. This might be interpreted as a localized area of hypertrophy, but in my opinion is probably fibroadenomatous (fig. 70).

Of 5 cases of chronic myeloid leukemia, 2 (or 40%) showed deposits in breast; of 3 cases of lymphatic leukemia (1 aleukemic) none showed deposits; 2 cases of acute monocytic leukemia showed deposits; 1 case of rare eosinophilic leukemia showed deposits; none of 3 cases of myelomatosis showed deposits, and of 8 cases of lymphoid neoplasm, 3 showed deposits. Two of these latter were of Hodgkin's type and the other was a lymphosarcoma. In the leukemias the infiltrates were most frequently periductal but sometimes perivascular also. In the lymphosarcoma case, the infiltrate was also periductal, but in the instances of Hodgkin's disease the infiltrates were largely stromal and sometimes perivascular (figs. 74, 75, and 76).

CONCLUSIONS

Females.—At autopsy about 50 percent of women showed a "normal" appearance of the breast, if we regard lobular adenosis in the younger woman and atrophy in the older woman as natural phenomena.

Lobular adenosis does, however, continue in some women after the menopause, possibly due to continued hormonal production in some site other than the ovary. In younger women adenosis was more often seen when the ovarian stroma was of high cellularity and when follicular or luteal activity was evident. The incidence of adenosis was not affected by the amount of fibrous or fatty tissue in the breast stroma.

Sclerosing adenosis occurred as the only abnormality in some postmortem female breasts but often also accompanied other changes including epitheliosis. The over-all incidence was 7.0 percent of cases examined. It usually occurred in older women and might be seen at any phase of development. Only 25 percent of cases were bilateral in this study.

Fibroadenoma was an infrequent finding at autopsy.

Epitheliosis, *i.e.*, epithelial hyperplasia, was seen as the sole proliferative lesion in 12.9 percent of cases but the over-all incidence was 22.0 percent. It was not common under age 40. Epitheliosis appeared to be more frequent in fatty than in fibrous breasts. The most common site was in the ducts only and the most common pattern was papillary. The majority of cases were mild, 25 percent moderately severe, and 6.8 percent were marked in degree.

Papillomatosis and papilloma were infrequent and were seen in only 2.9 percent of all cases.

Neither in epitheliosis nor in papillomatosis was any constant, consistent feature noted in ovary or pituitary.

Of some 40 cases of carcinoma studied, only 6 were of occult type. This is a small number of occult neoplasms, *i.e.*, 6 in 766 cases compared with occult carcinoma of prostate, where various workers claim to find much higher rates. Secondary spread to the contralateral breast occurred in 10 of 34 clinical cases, in about 30 percent. Epitheliosis was present in the breast of primary origin in 10 of 16 or in 63 percent. The opposite breast, studied in all 40 cases, was atrophic in 47.5 percent, showed adenosis in 22.5 percent, and epitheliosis in 30 percent.

In 148 cases of the series the cause of death was malignant neoplasia other than breast carcinoma. When these cases were analyzed the only significant feature was: In the group of cancers of the female genitalia there was a higher incidence of proliferative change in the breast than in the other groups. Secondary deposits were noted in carcinoma of pancreas, bronchus, in Hodgkin's disease, leukemia, and in acute histiocytic medullary reticulosis.

The state of the breast in some well-defined disorders, alleged to have endocrine significance, was also analyzed: No significant features were noted in diabetes mellitus, hyperostosis frontalis interna, or hepatic cirrhosis. Women dying of hypothyroidism appeared to have a higher incidence of parenchymal atrophy than was normal. This was true also of Simmonds' disease but not of Addison's disease. However, the numbers considered here are small.

In 275 cases an attempt was made to examine the ovaries and pituitary. No significant relationships were discovered, but interesting figures on the incidence of some phenomena were noted in both ovary and pituitary. Neither oophorectomy nor considerable destruction of the pituitary by radon implant necessarily led to atrophy of the breast parenchyma.

As regards breast types: In younger women the breasts were nearly always largely fibrous, but in many middle-aged women fatty infiltration was noted. In very old women, however, the breasts were usually fibrous and quite atrophic. In a few older women, the virginal appearance was maintained without gross atrophy or fatty change.

Just over 70 percent of all women autopsied showed either some duct dilatation or formation of microcysts, and about 40 percent showed some degree of pale apocrine-cell metaplasia. About 16 percent showed some degree of periductal hyperplasia of elastic tissue but only 9 percent showed desquamation of colostrum corpuscles. Lobules showing persistent secretory change, sometimes long after the menopause, were noted in nearly 4.0 percent of all women. About 23.0 percent of all women showed some degree of periductal or intralobular round-cell infiltrates, but no examples of plasma-cell mastitis were seen. Mammalithiasis was rare, seen in only 0.5 percent.

Males.—Of 500 examined only 3 percent showed the appearance of gynecomastia. Of these 15 cases, 3 showed an unusual degree of epithelial hyperplasia, and this was noted in an additional 8 cases without stromal hyperplasia. Among the most common causes were diethylstilbestrol therapy, hepatic carcinomatosis, and continued venous congestion of liver. Hepatic cirrhosis was accompanied by hypertrophy in only 2 of 12 cases.

As regards neoplasia, 1 man showed mammary carcinoma with Paget's disease of the nipple. One case each had secondary melanoma and secondary bronchial carcinoma. Deposits of leukemic cells and of Hodgkin's disease and lymphosarcoma were noted.

One male showed a small fibroadenomatous area.

Among the other findings were subareolar apocrine glands in 52.2 percent of cases, duct ectasia in 6 percent, and senile thickening of the basement membrane in 7.2 percent.

These results are presented in the full realization of the imperfections of this work. There can be no doubt that these are minimal incidences that could be raised by the study of more numerous blocks of tissue. A counsel of perfection to any future student of the problem would be to make serial sections of the entire breast, but this seems unlikely ever to be achieved under present conditions.

My only concrete claim is that in the postmortem breast, presumably symptom-free, I have shown a wide variety of appearances, often thought to be restricted to the surgically removed diseased organ. The incidence of mammary epitheliosis in the female population would allow an adequate reservoir for the development of neoplasia, but an obvious fact is that many women with epitheliosis of the breast die without developing malignant disease. These findings perhaps contain a warning against too enthusiastic advocacy of simple mastectomy except for very gross epitheliosis in any mammary biopsy.

SUMMARY

At autopsy, the breasts of 800 women and 500 men were examined microscopically with special reference to proliferative changes of importance in human surgical pathology.

The minimal incidence of lobular adenosis, sclerosing adenosis, epitheliosis, and papillomatosis was established in the female.

A number of cases of carcinoma of the breast, both clinical and occult, was studied with special reference to the contralateral mammary gland.

Some attention was given to gross breast types, the question of normality and atrophy, the incidence of cysts and other phenomena, *e.g.*, apocrine metaplasia, periductal elastic hyperplasia, formation of colostrum corpuscles, persistence of secretion, change, mammalithiasis, etc.

In a proportion of cases the ovaries and pituitary were studied. In these the incidence of certain phenomena was ascertained, but no clear-cut histological relationships between breast, ovary, and hypophysis were established. The condition of the breast in certain endocrine diseases was investigated, as was the appearance of the breast in women dying of malignant disease other than breast carcinoma.

In the male, some aspects of the breast structure, *e.g.*, presence of subareolar apocrine glands, ectasis of ducts, presence of tactile corpuscles and of lymphocytes, etc., were studied. The occurrence of breast hypertrophy, epithelial hyperplasia, and neoplasia was discussed. Some attention was given to the state of the liver in mammary hypertrophy in the male.

Under each heading the literature was briefly reviewed.

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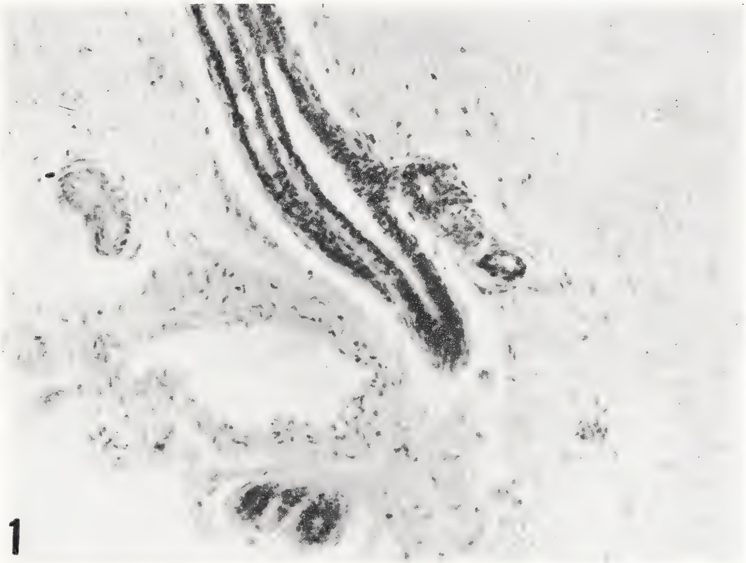
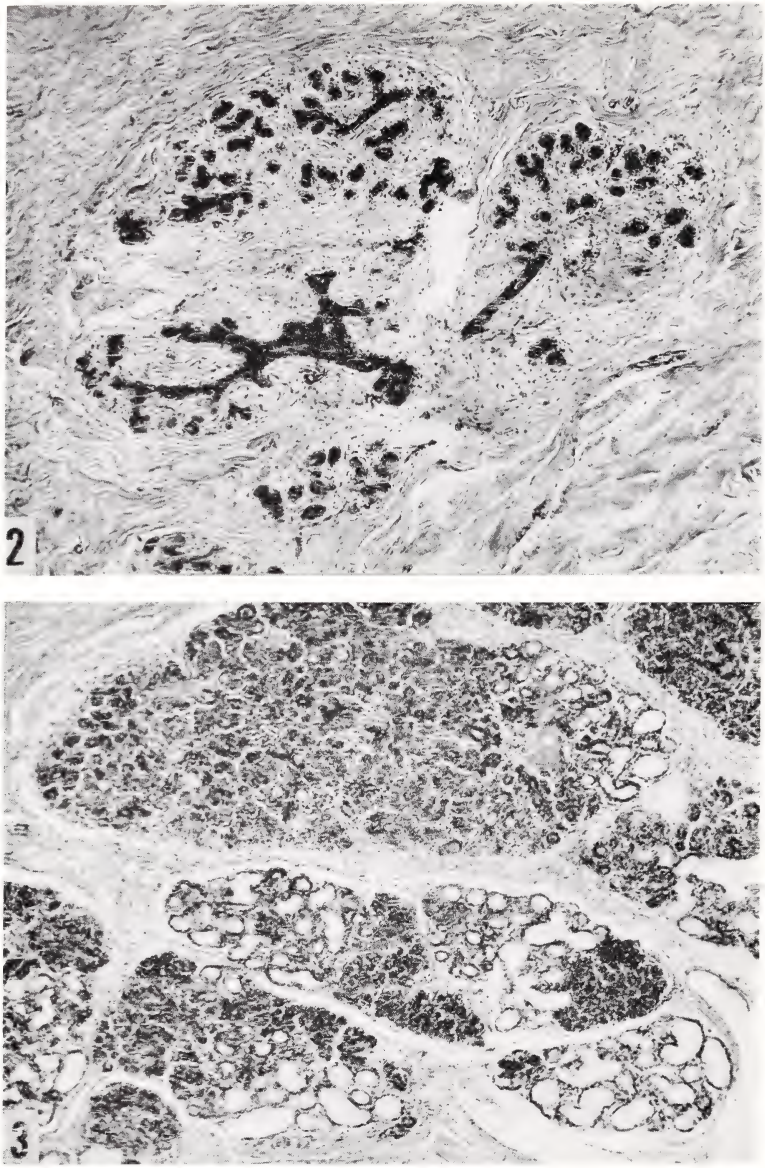
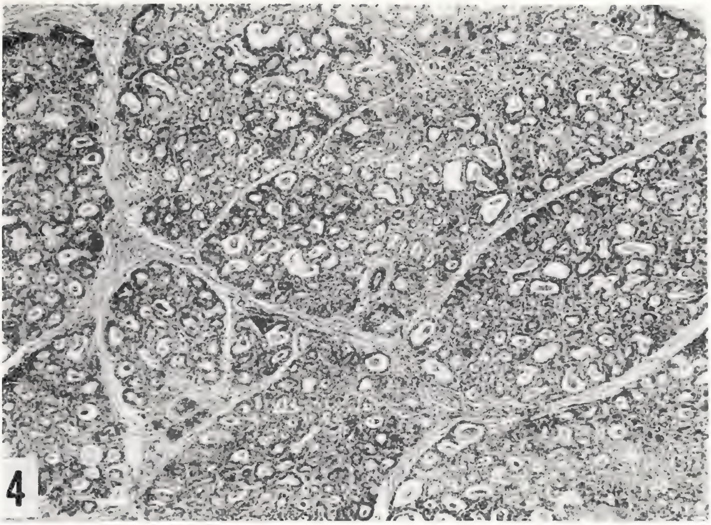


FIGURE 1.—Atrophic duct without lobules in breast of an 84-year-old woman. The collagen is hyalin and refractile.
Hemalum and eosin. × 130. A 9923.

SANDISON



FIGURES 2 and 3.—Two lobules from the breast of a 20-year-old woman. This illustrates the changes produced in physiological adenosis.
Hemalum and eosin. × 60. A 8445.



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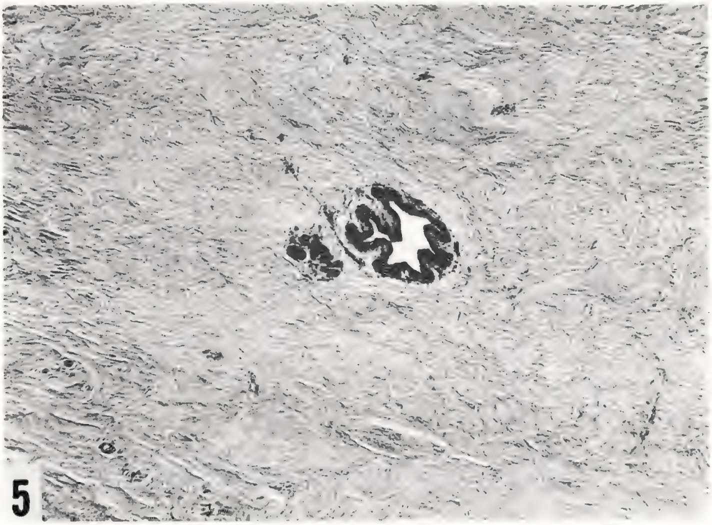

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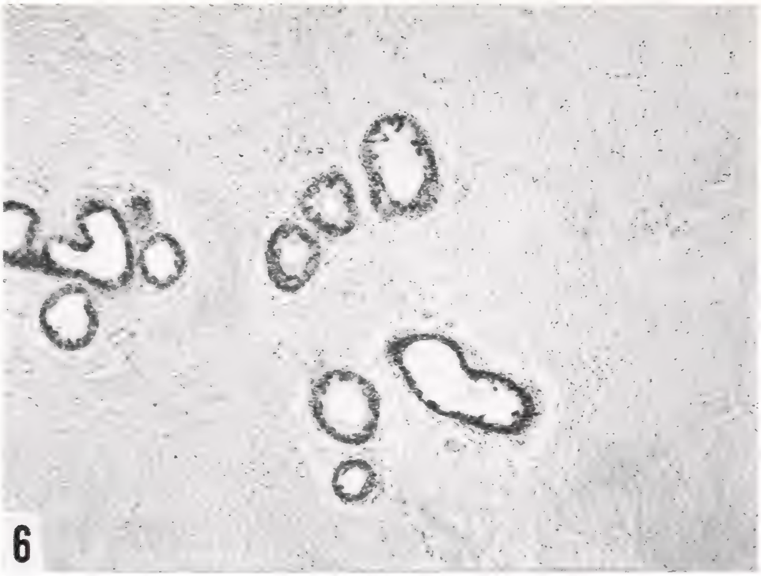
Figure 4.—Pregnancy adenosis in a 33-year-old woman dying of accidental hemorrhage at term.
Hemalum and eosin. × 45. A 9915.

Figure 5.—Failure to develop lobules in a 20-year-old woman with tuberculosis and diabetes. This resembles the premenarche appearance (*cf.* fig. 6).
Hemalum and eosin. × 45. A 9829.



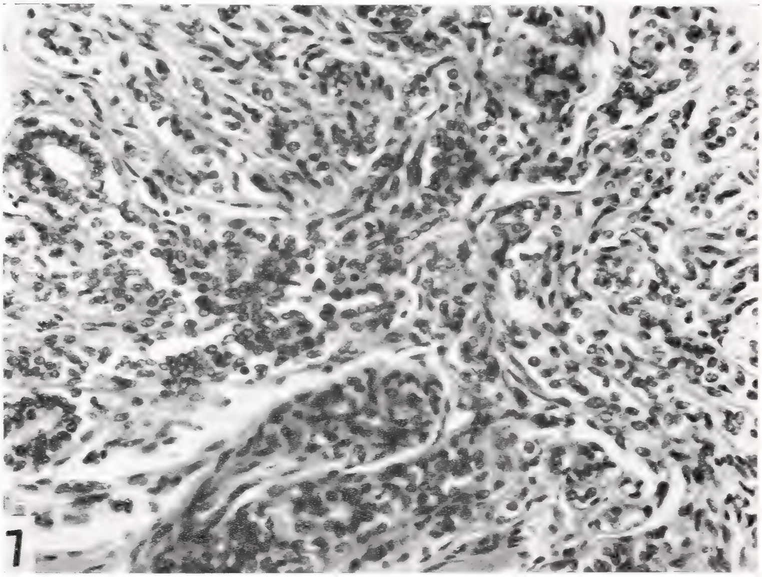
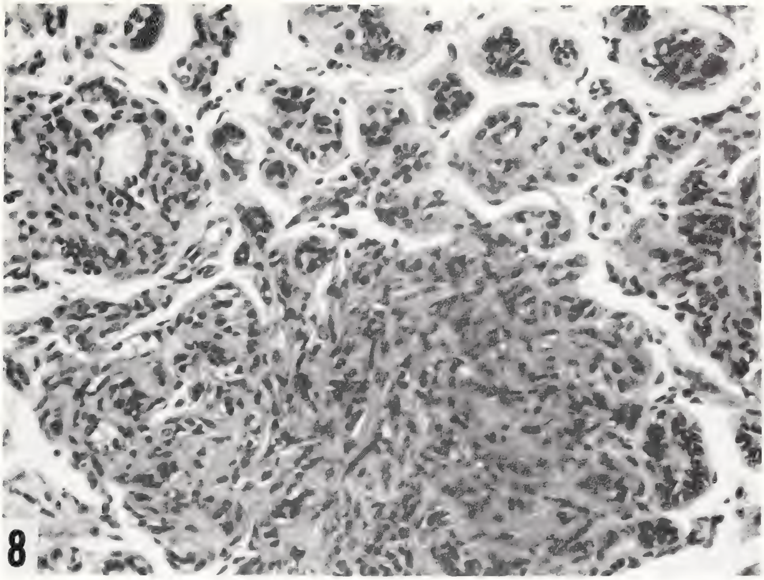


FIGURE 6.—Prepubertal breast in a 14-year-old girl for comparison with figure 5. Hemalum and eosin. × 60. A 7792.

FIGURE 7.—High-power view of breast lobule in the florid stage of sclerosing adenosis.
Hemalum and eosin. × 250. A 9562.

SANDISON



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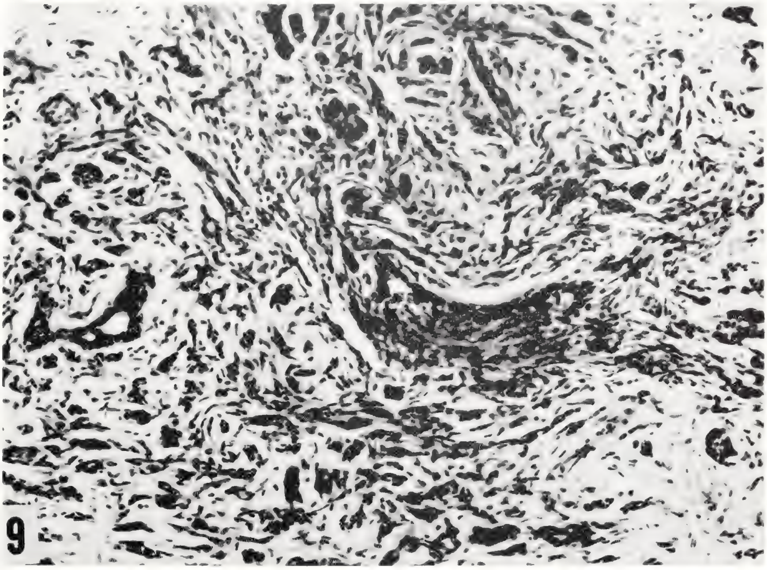

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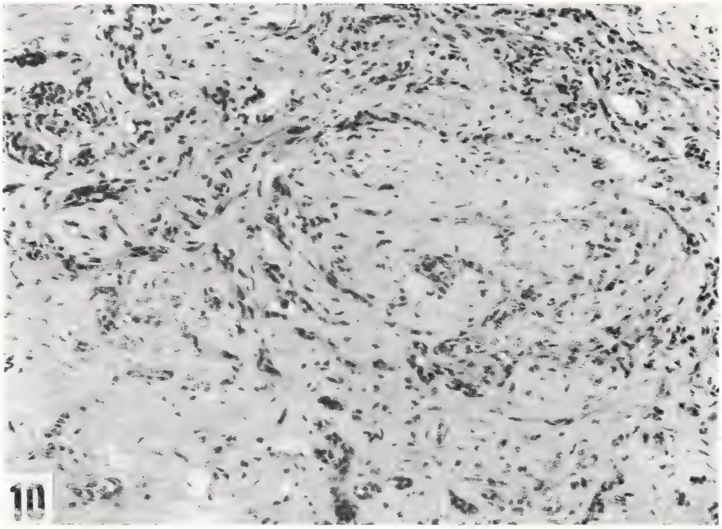
Figure 8.—Another case of sclerosing adenosis in the intermediate stage.
Hemalum and eosin. × 250. A 9540.

Figure 9.—Lower-power view of sclerosing adenosis becoming more sclerosed and suggestive of scirrhous neoplasm.
Hemalum and eosin. × 130. A 9722.



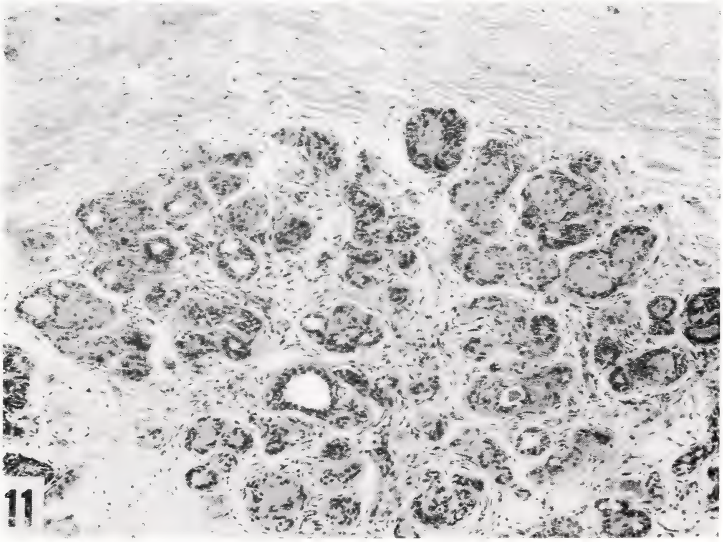
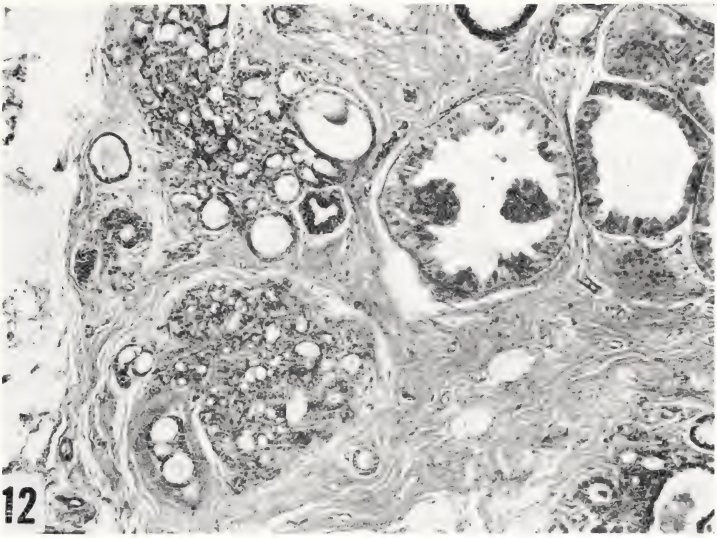


FIGURE 10.—Low-power view of sclerosed sclerosing adenosis showing the lobular nature of the change—an important diagnostic point.
Hemalum and eosin. × 45. A 8062.

FIGURE 11.—A hyalinized lobule which has not undergone previous proliferation for comparison with the foregoing example of sclerosing adenosis.
Hemalum and eosin. × 100. A 9628.



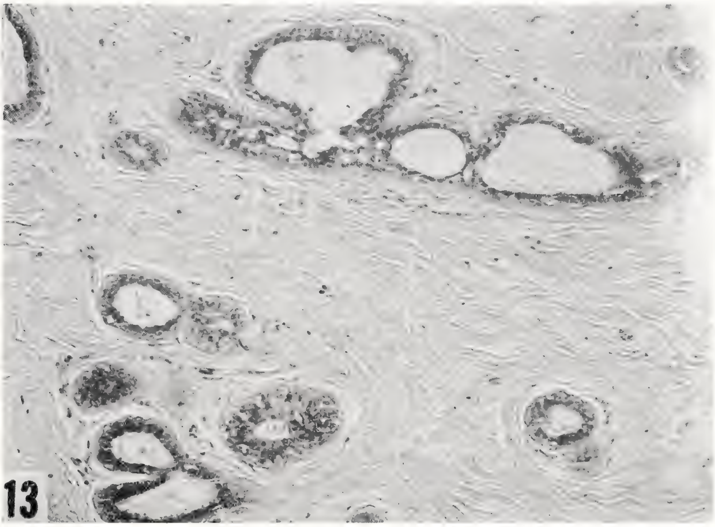
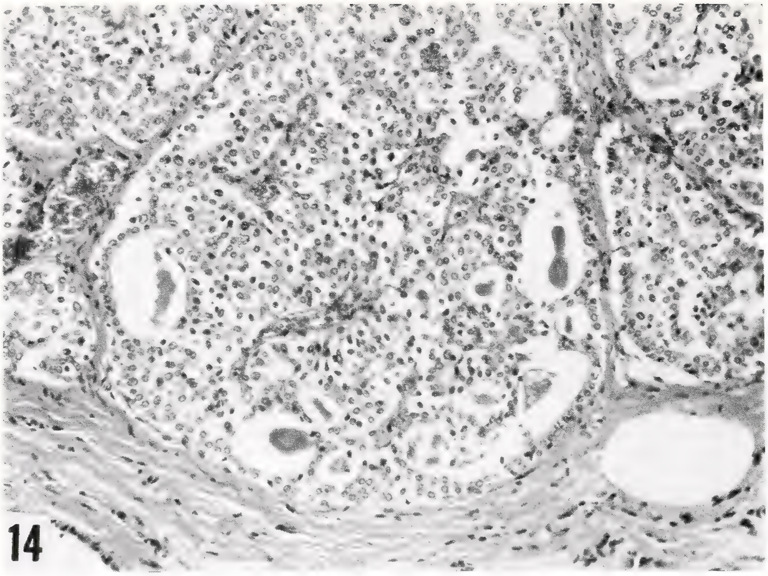


FIGURE 12.—A focus of sclerosing adenosis associated with microcysts and apocrine change in the breast of a 64-year-old woman.
Hemalum and eosin. × 85. A 8568.

FIGURE 13.—Fibroadenoma in a 74-year-old woman—a small 1.0 cm diameter tumor with hyalinized stroma.
Hemalum and eosin. × 130. B 0219.



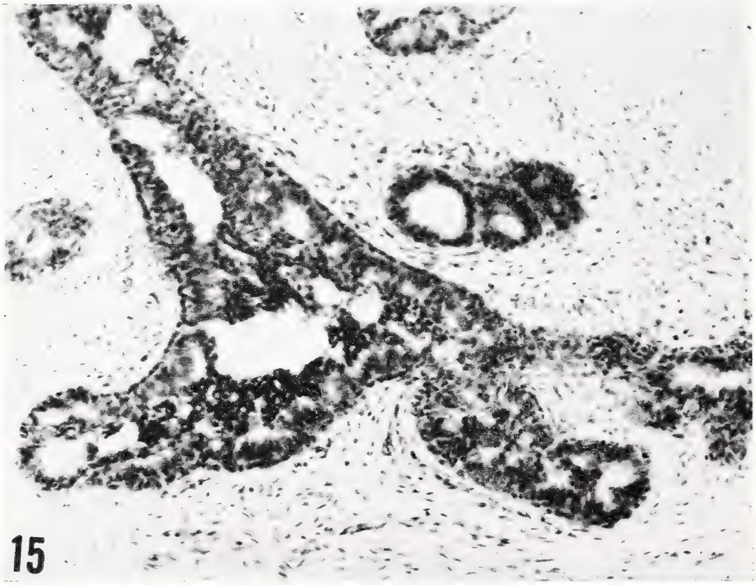
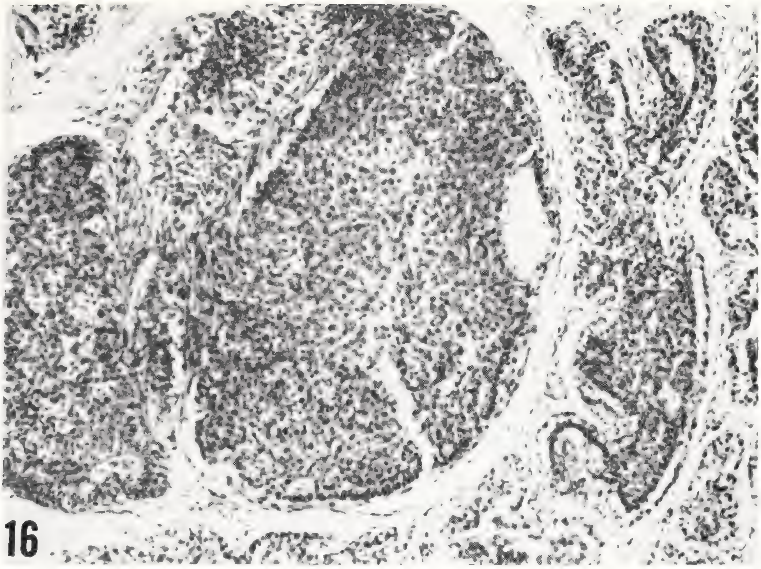


FIGURE 14.—Mild cribriform epitheliosis in a 67-year-old woman. Hemalum and eosin. × 130. A 9856.

FIGURE 15.—Epitheliosis of ducts and acini in a 44-year-old woman; the cells are more darkly staining than those in figure 14.
Hemalum and eosin. × 130. A 9722.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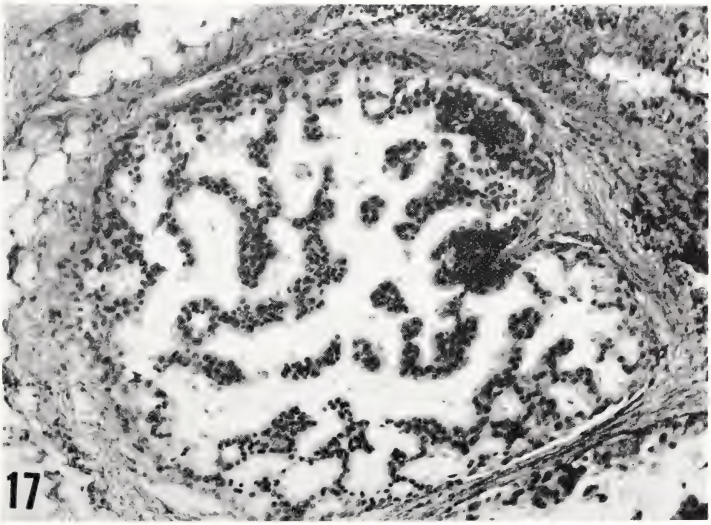
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Figure 16.—Marked intraductal and intra-acinar epitheliosis in a 73-year-old woman; of solid type.
Hemalum and eosin. × 130. A 9537.

Figure 17.—Complex papillary intraductal epitheliosis in a 43-year-old woman with comedo intraductal carcinoma elsewhere.
Hemalum and eosin. × 130. A 9986.

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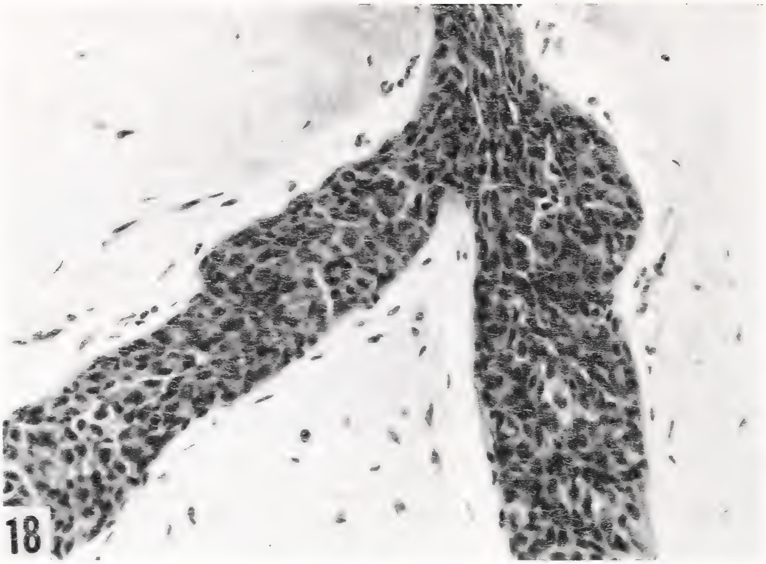


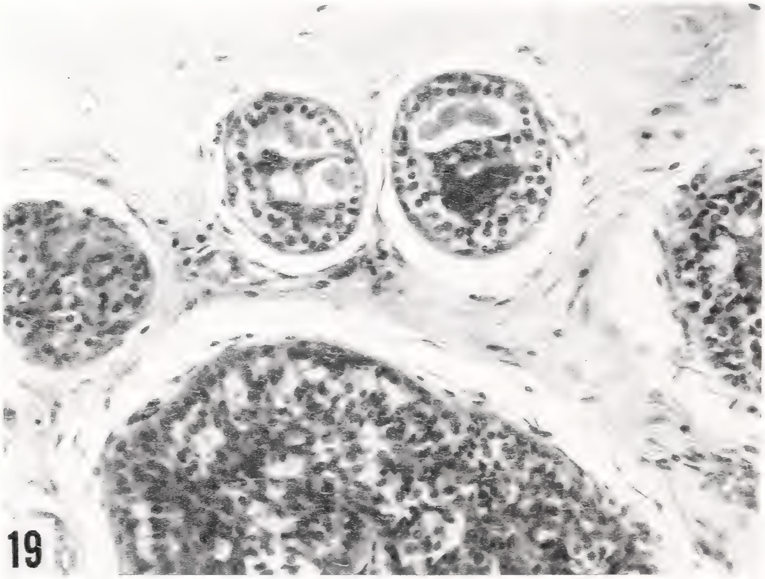
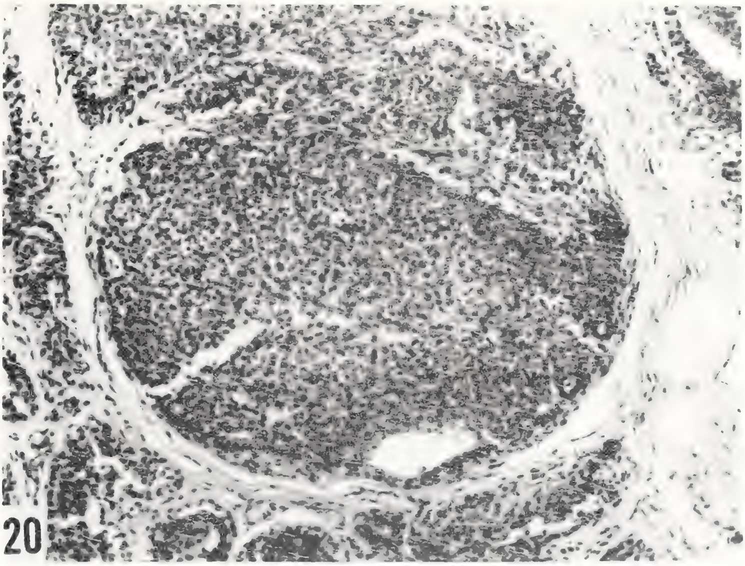


FIGURE 18.—Intraductal epitheliosis of solid type with hyperchromatic cells in a 62-year-old woman.
Hemalum and eosin. × 250. A 9823.

FIGURE 19.—Intraductal epitheliosis of cribriform type in the fibrofatty breast of a 73-year-old woman.
Hemalum and eosin. × 230. A 9537.



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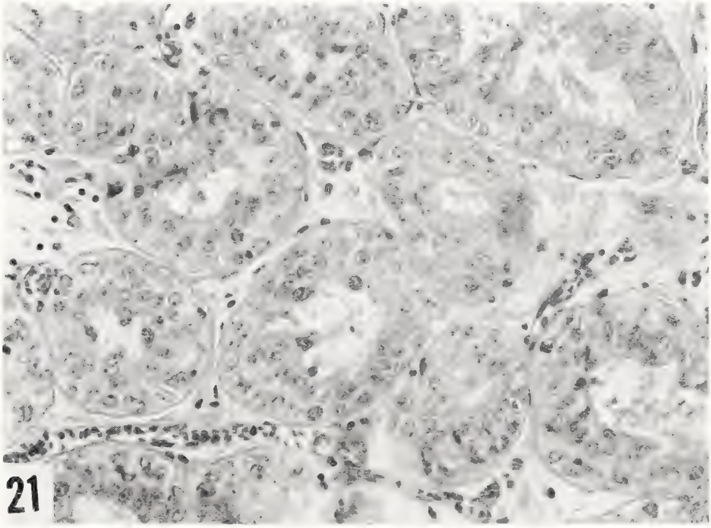

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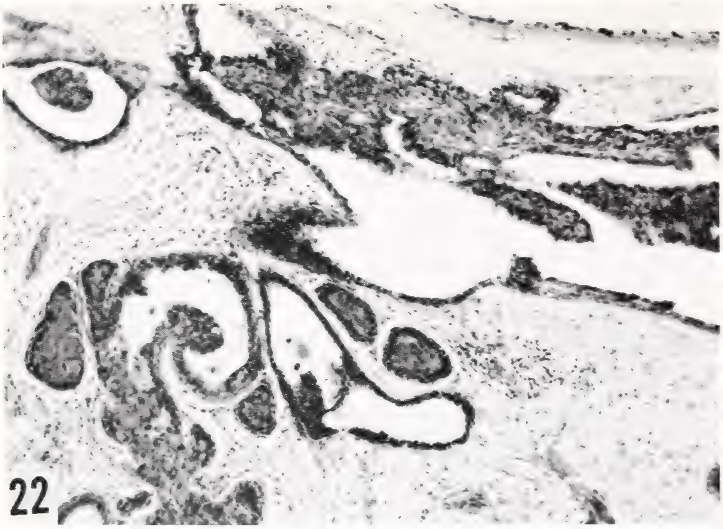
FIGURE 20.—Solid intraductal epitheliosis with myoepithelial component, in the same case.
Hemalum and eosin. × 130. A 9537.

FIGURE 21.—Intra-acinar epitheliosis in a 71-year-old woman.
Hemalum and eosin. × 230. A 9689.



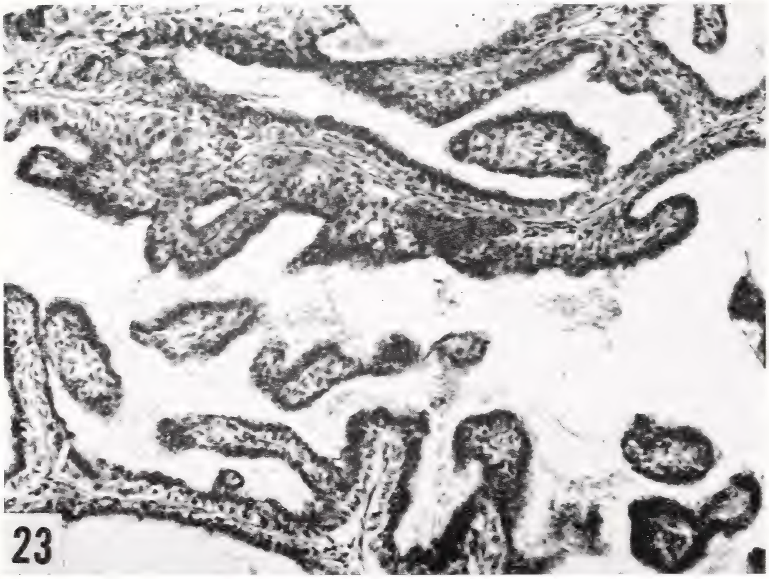
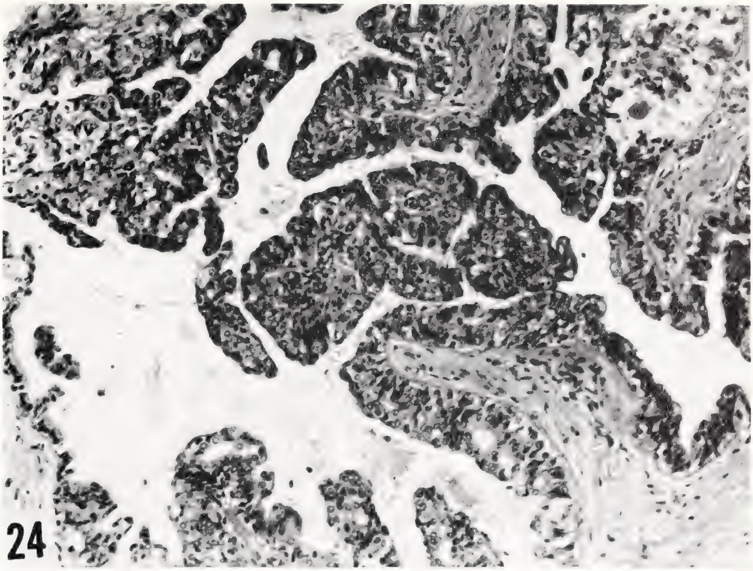


FIGURE 22.—A small intraductal papilloma in a 42-year-old woman who also showed papillary epitheliosis.
Hemalum and eosin. × 130. A 9812.

FIGURE 23.—Papillomatosis in a 73-year-old woman who also had papillary epitheliosis. The fibrovascular cores and hyperchromatic epithelial nuclei are shown.
Hemalum and eosin. × 130. A 8126.



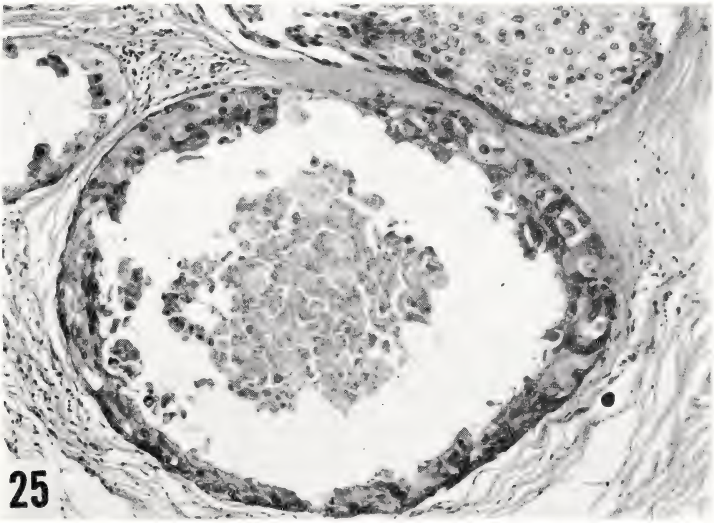


FIGURE 24.—Complex papilloma in duct of a 67-year-old woman.
Hemalum and eosin. × 130. A 9856.

FIGURE 25.—Occult intraductal carcinoma in a 71-year-old woman. Central necrosis ("comedo carcinoma") is seen.
Hemalum and eosin. × 130. B 0143.
(Courtesy E. and S. Livingstone, Edinburgh)

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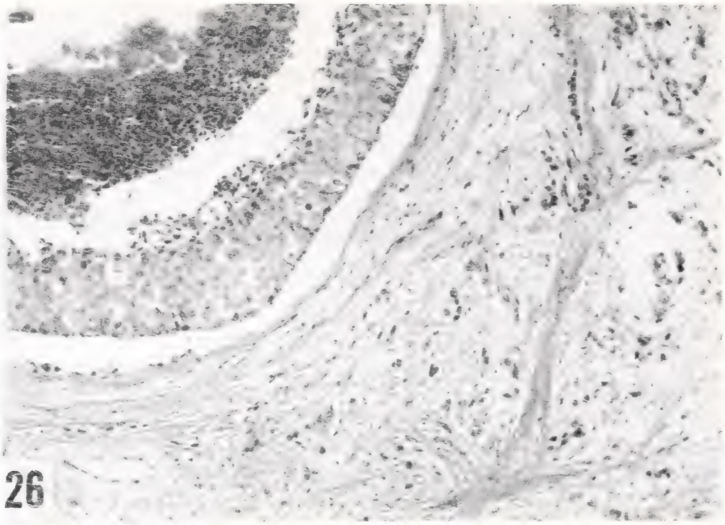


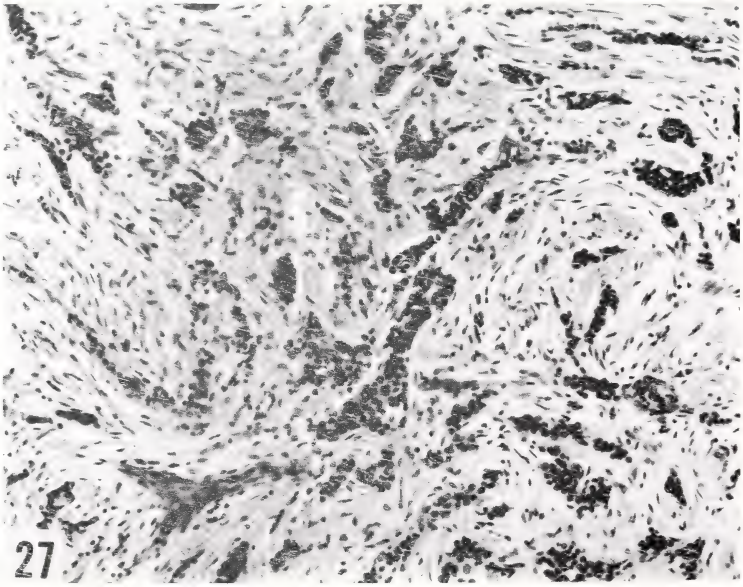
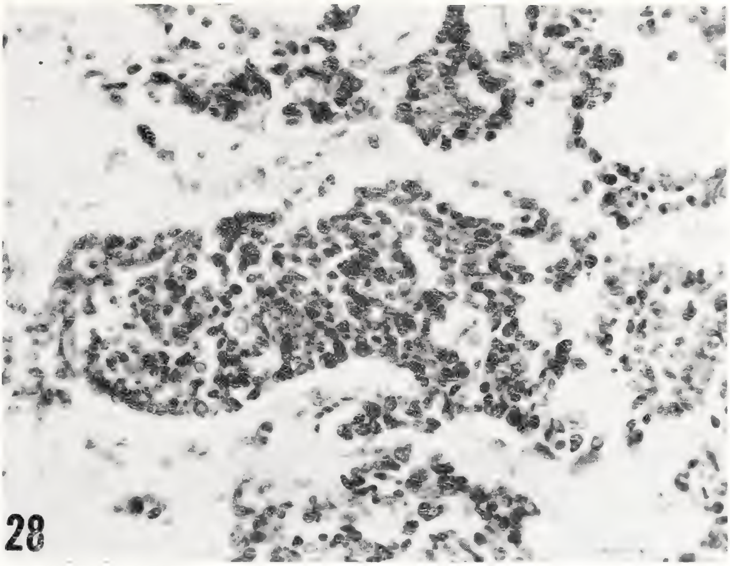


FIGURE 26.—Intraductal and infiltrative carcinoma in a 31-year-old woman recently pregnant. Hemalum and eosin. × 130. A 9719.

FIGURE 27.—Secondary carcinoma in breast, from a primary in the contralateral breast of a 42-year-old woman.
Hemalum and eosin. × 130. A 9751.



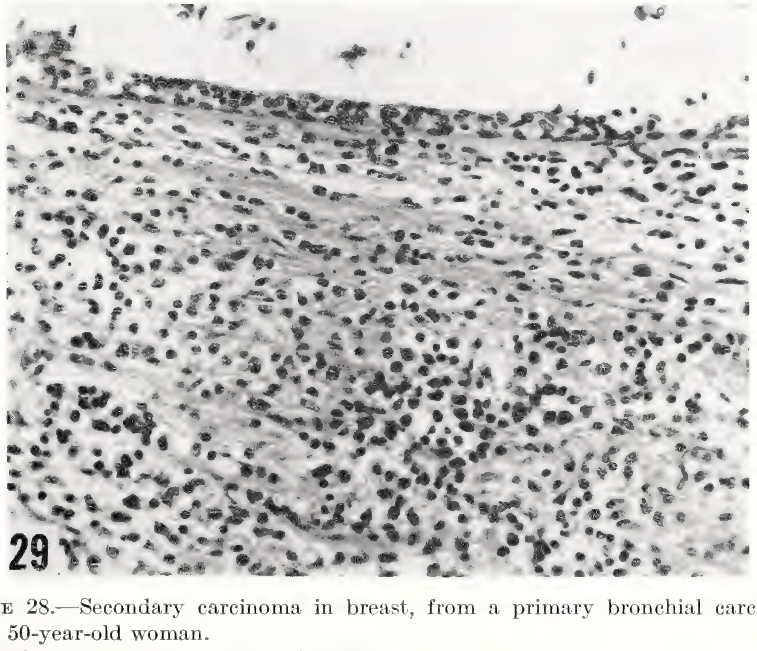
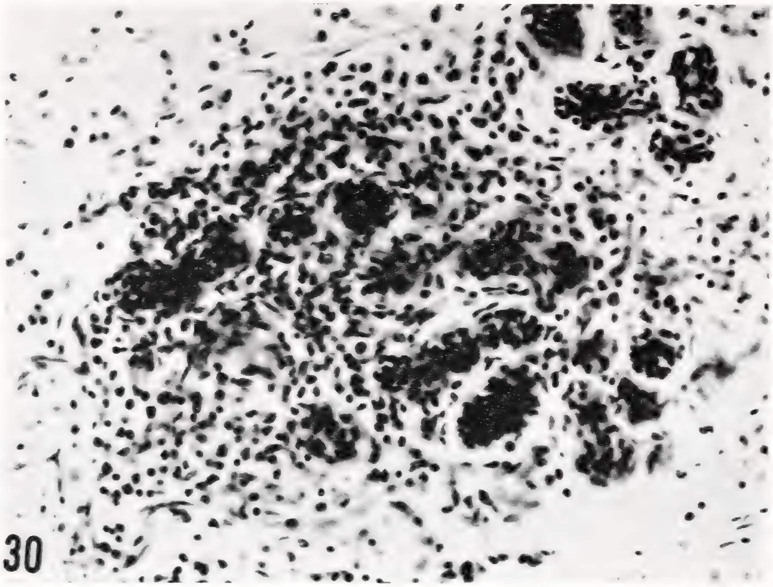


Figure 28.—Secondary carcinoma in breast, from a primary bronchial carcinoma in a 50-year-old woman.
Hemalum and eosin. × 250. A 9902.

Figure 29.—Periductal leukemic deposits in a 55-year-old woman with acute leukemia.
Hemalum and eosin. × 250. A 7941.



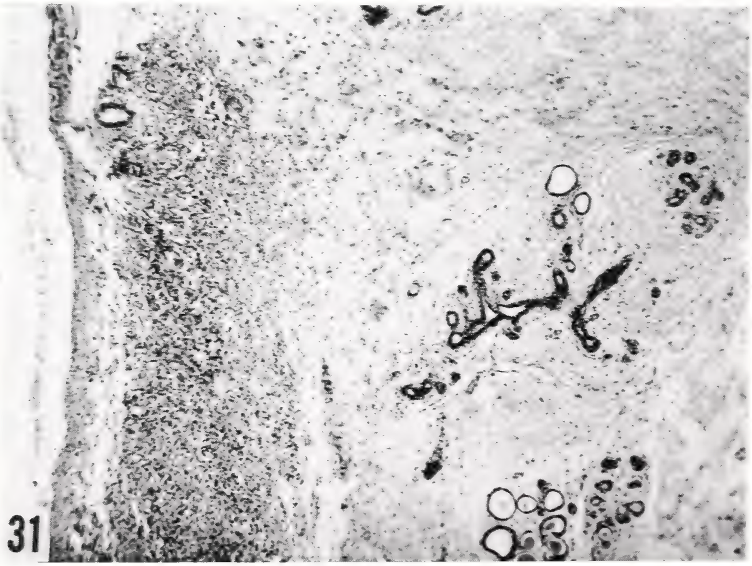
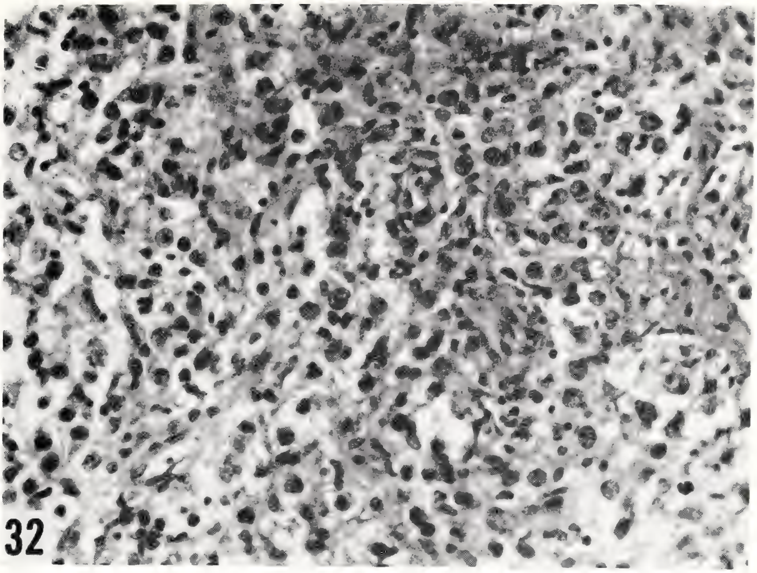


FIGURE 30.—Breast lobule of a 30-year-old woman with acute lymphoblastic leukemia showing infiltrate.
Hemalum and eosin. × 250. A 9624.

FIGURE 31.—Low-power view of Hodgkin's tissue in the breast of a 30-year-old woman: the infiltrate is not lobular.
Hemalum and eosin. × 45. A 7771.



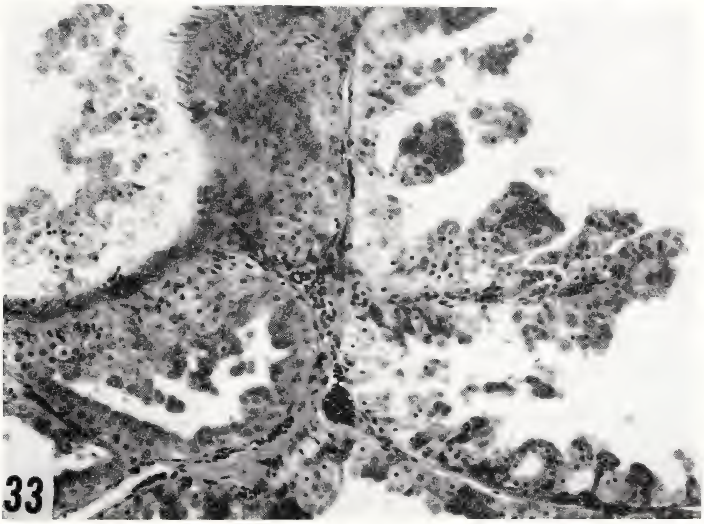
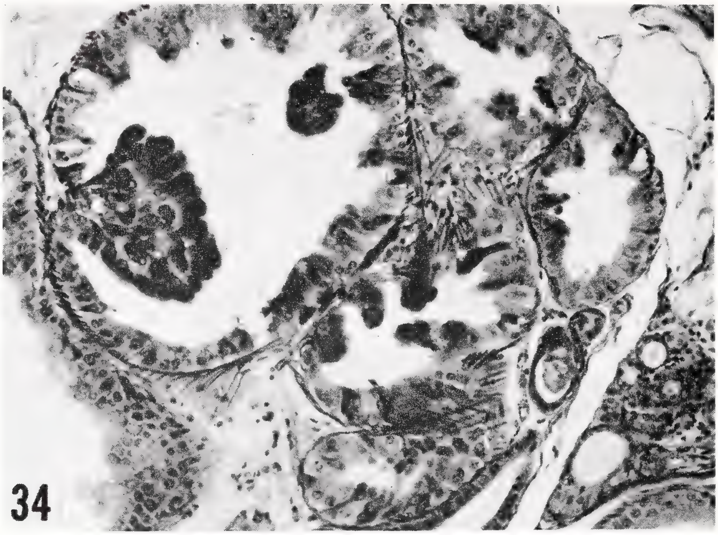


FIGURE 32.—High-power view of figure 33 to show cellular infiltrate. Hemalum and eosin. × 250. A 7771.

FIGURE 33.—Apocrine change with papillary intracystic projections. Hemalum and eosin. × 130. A 9488.



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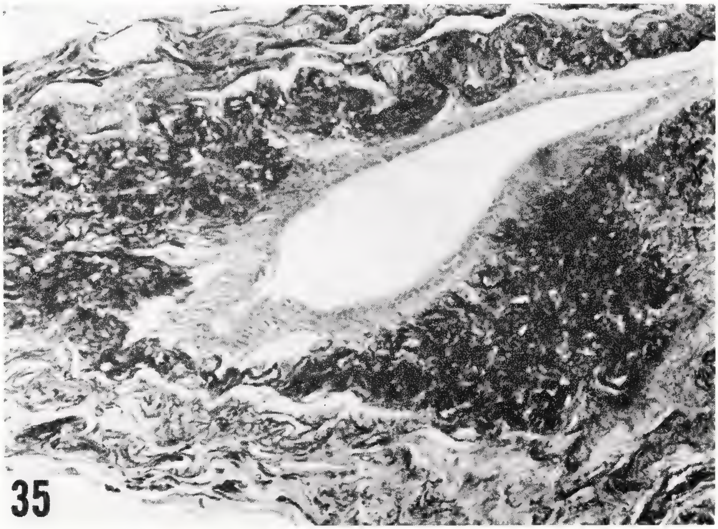

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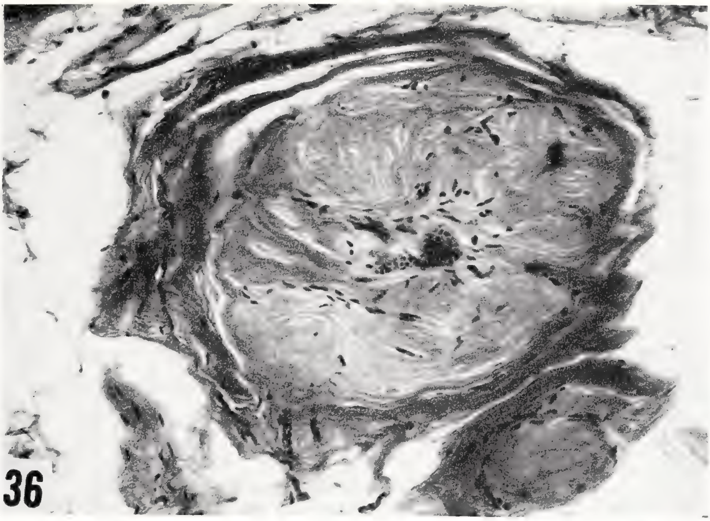
FIGURE 34.—Apocrine change to show myoepithelium stained by phosphotungstic acid-hematoxylin. × 130. A 8568.

FIGURE 35.—Periductal elastic hyperplasia ("elastosis") in atrophic breast of a 60-year-old woman.
Weigert's elastica stain. × 130. A 9524.



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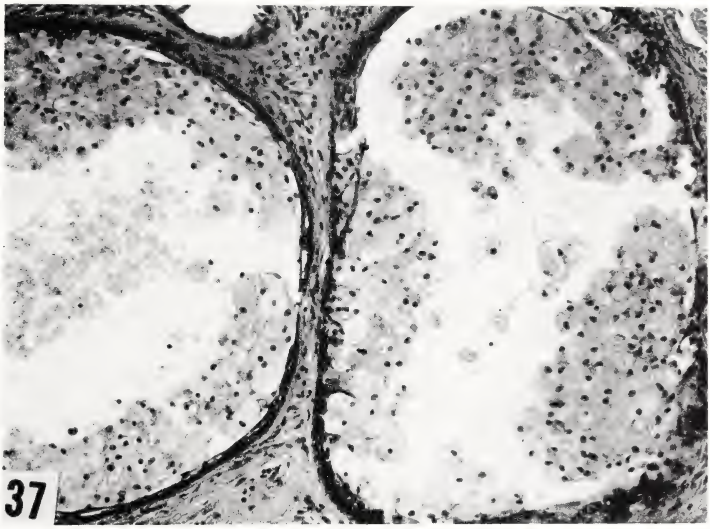

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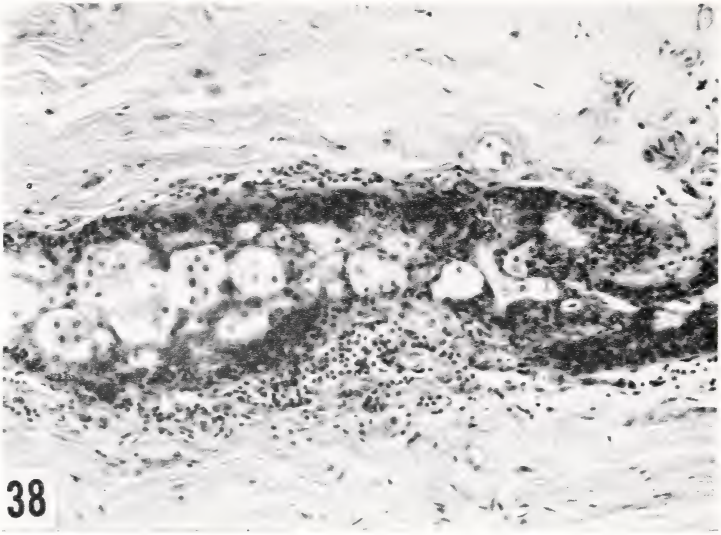
FIGURE 36.—Another duct from the same breast showing periductal elastosis which has virtually obliterated the duct.

Hemalum and eosin. × 130. A 9524.

FIGURE 37.—Intracystic desquamation of "colostrum corpuscles" in a 64-year-old woman.

Hemalum and eosin. × 130. B 0109.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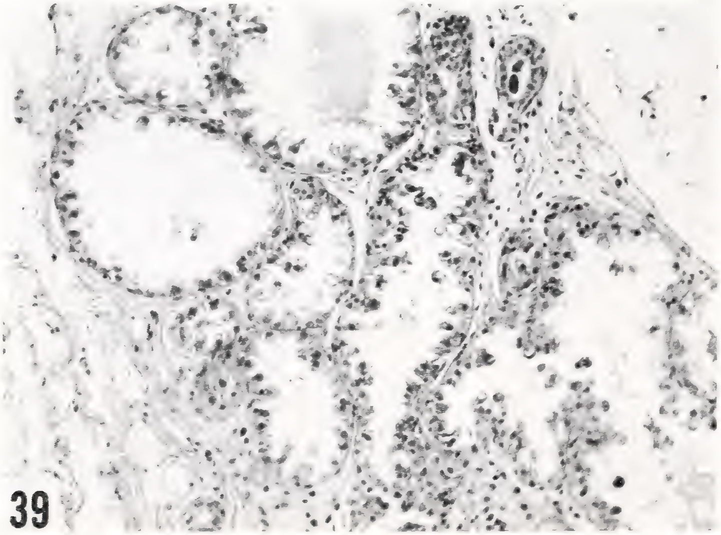
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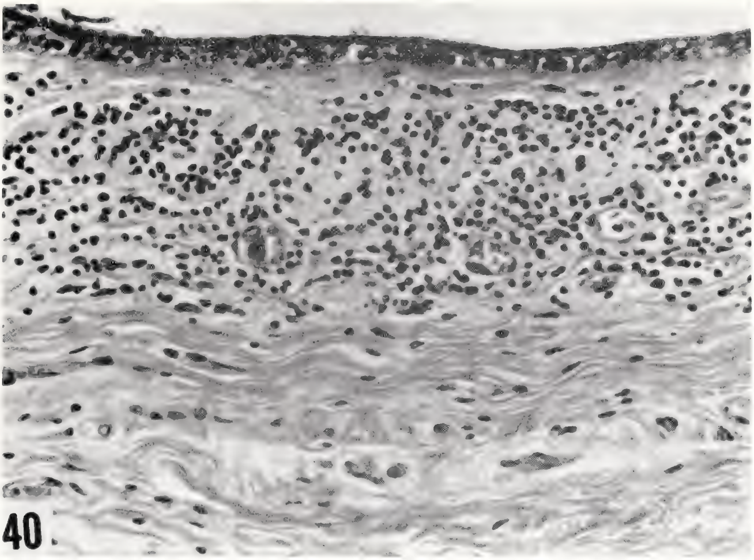
FIGURE 38.—Cystiphorous desquamative epithelial hyperplasia in a 58-year-old woman of type described by Cheatle and Cutler (1931). There is also periductal lymphocyte infiltration.
Hemalum and eosin. × 130. B 0003.

FIGURE 39.—Persistence of secretory effect of lactational type in a 68-year-old woman with otherwise atrophic breast parenchyma.
Hemalum and eosin. × 130. A 8470.



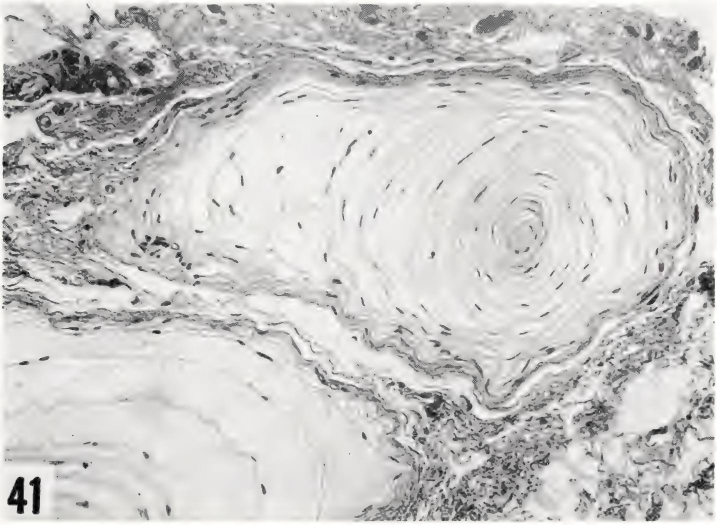
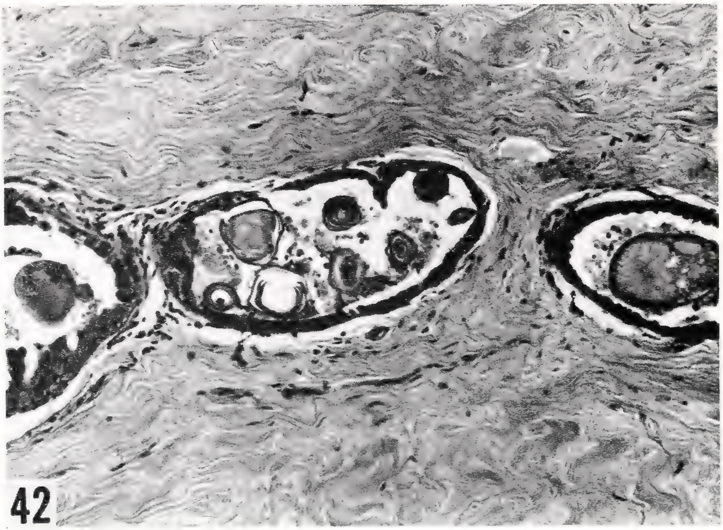


FIGURE 40.—Periductal lymphocyte infiltrates in a 64-year-old woman with atrophic breasts. Hemalum and eosin. × 130. A 9688.

FIGURE 41.—Pacinian corpuscle or tactile nerve ending of type seen in both male and female breasts.
Hemalum and eosin. × 130. A 9498.



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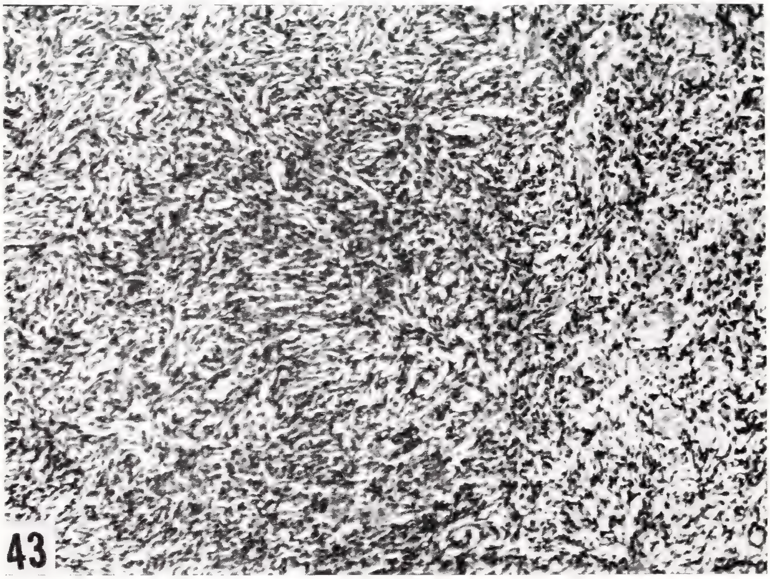

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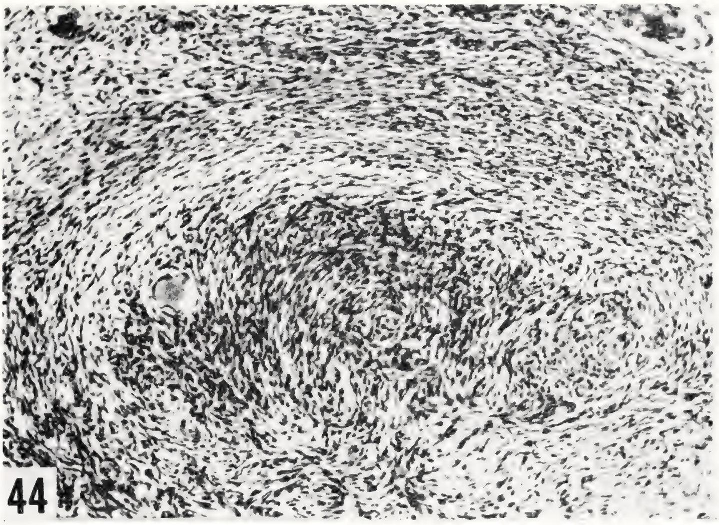
Figure 42.—Mammalithiasis (intraductal concretions) in a 70-year-old woman. Hemalum and eosin. × 130. A 9509.

Figure 43.—Highly cellular ovarian cortical stroma in a 62-year-old woman with atrophic breast parenchyma.
Hemalum and eosin. × 130. A 9547.
(Courtesy E. and S. Livingstone, Edinburgh)



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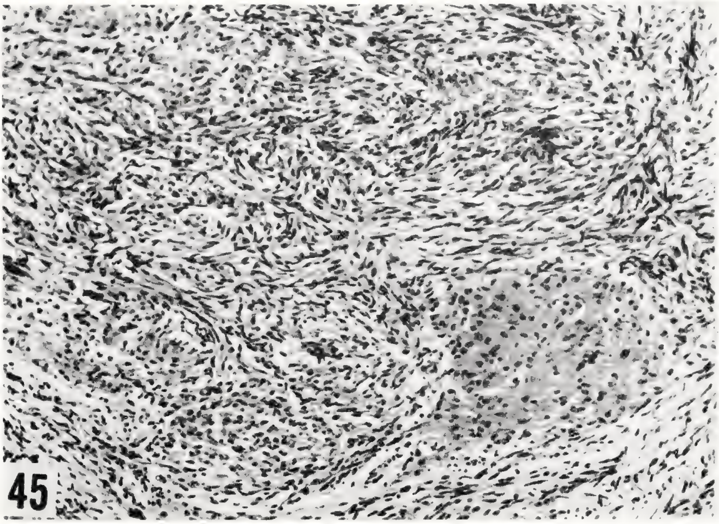

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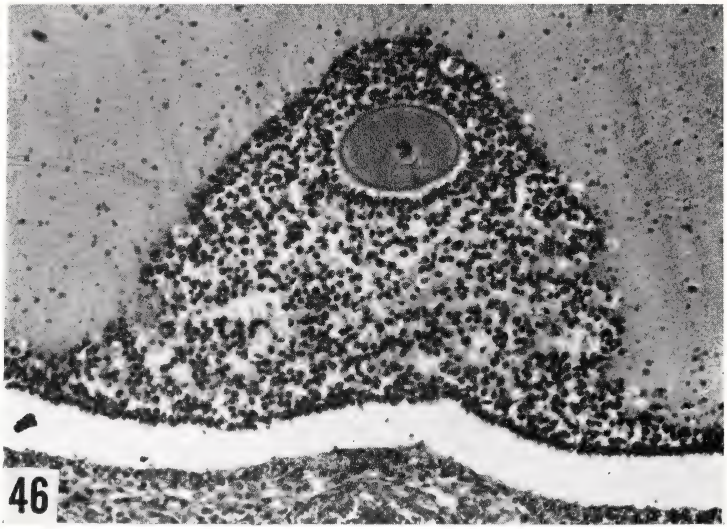
Figure 44.—Moderately cellular ovarian stroma in a 36-year-old woman with adenosis. Hemalum and eosin. × 130. A 9616.

Figure 45.—Less cellular ovarian stroma with some hilus cells in an 85-year-old woman with adenosis.
Hemalum and eosin. × 130. A 9606.
(Courtesy E. and S. Livingstone, Edinburgh)



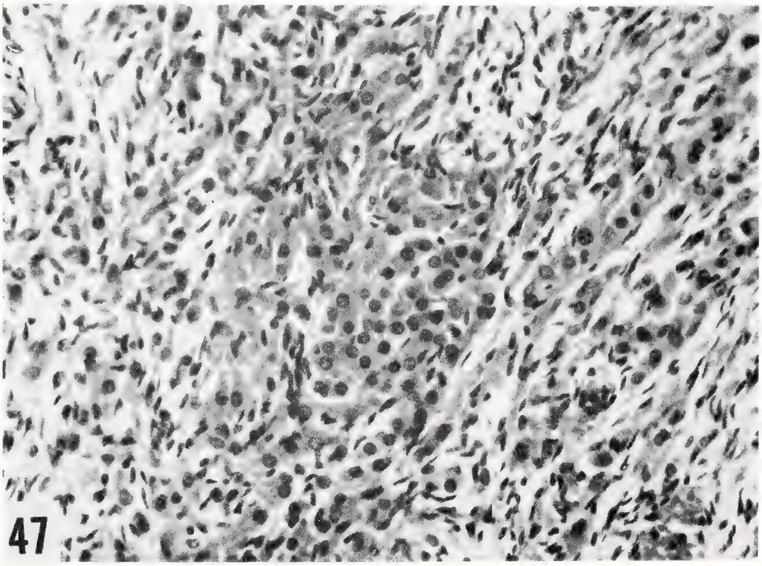
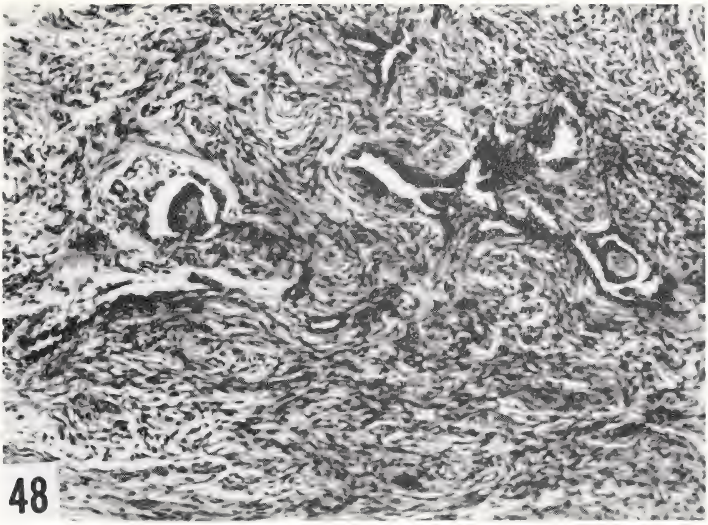


FIGURE 46.—Follicle formation in ovary of a 33-year-old woman with "normal" breasts.
Hemalum and eosin. × 130. A 9601.

FIGURE 47.—A group of interstitial cells at the hilum of the ovary of a 60-year-old woman.
Hemalum and eosin. × 250. A 9536.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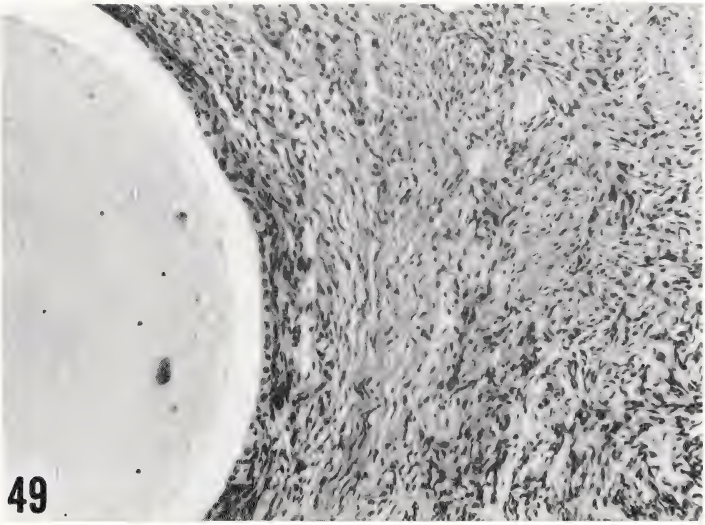
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FIGURE 48.—Vestigial tubular remnants at the hilum of the ovary of a 62-year-old woman.
Hemalum and eosin. × 130. A 9556.

FIGURE 49.—Simple cyst lined by cubical epithelium in the ovary of a 65-year-old woman.
Hemalum and eosin. × 130. A 9503.

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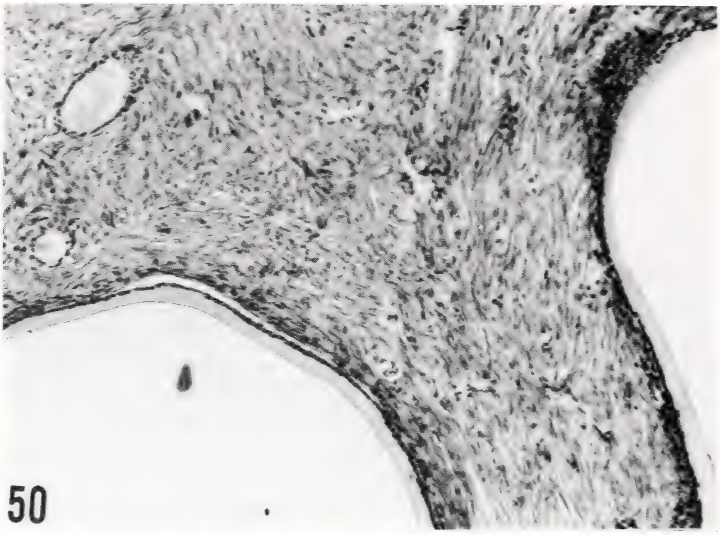

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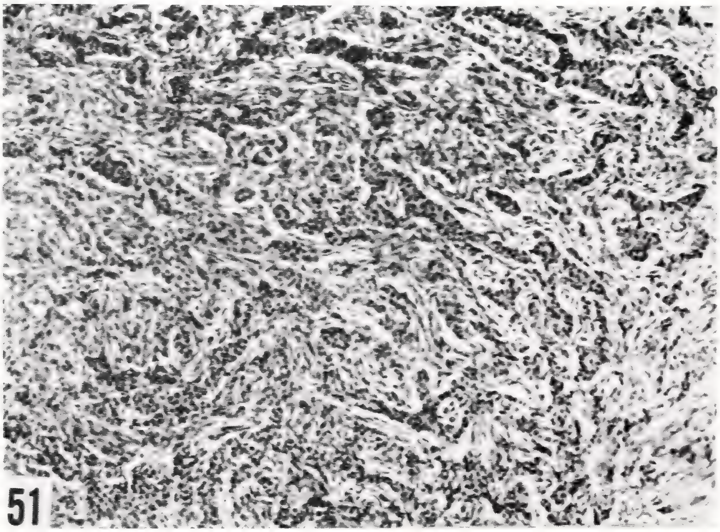

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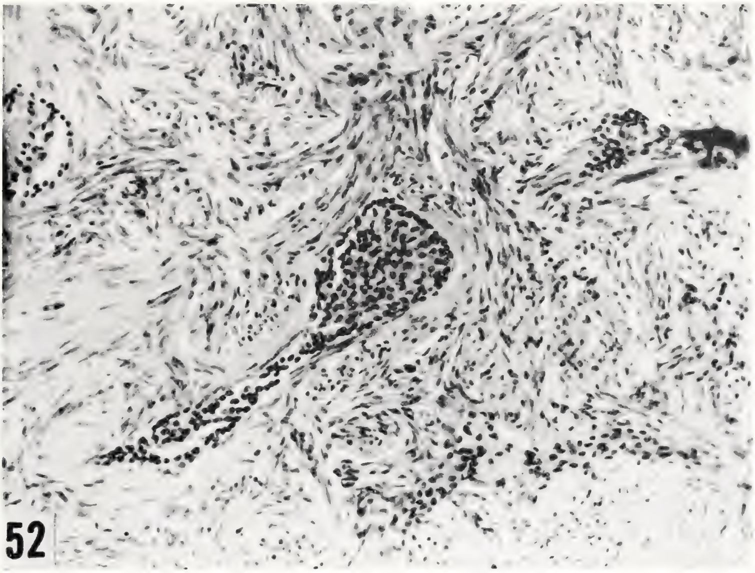
FIGURE 50.—Simple pseudo-mucinous cystadenoma in ovary of a 32-year-old woman with "normal" breasts.
Hemalum and eosin. × 130. A 9703.

FIGURE 51.—Invasive granulosa-cell ovarian tumor—an incidental finding in a 40-year-old woman with epitheliosis and sclerosing adenosis of breast.
Hemalum and eosin. × 130. A 9706.



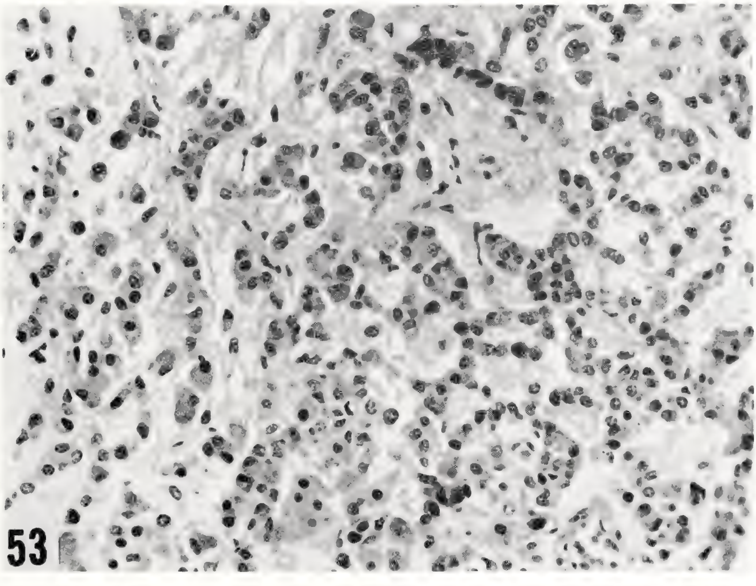
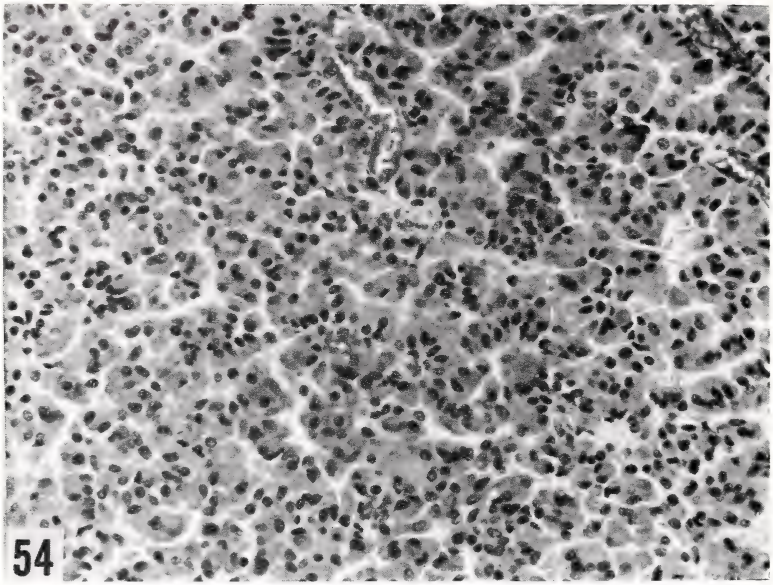


Figure 52.—Brenner tumor of ovary—an incidental finding in a 60-year-old woman with atrophic breasts.
Hemalum and eosin. × 130. A 9729.

Figure 53.—Scatter of beta cells into pars nervosa of pituitary of a 60-year-old woman.
Hemalum and eosin. × 250. A 9730.



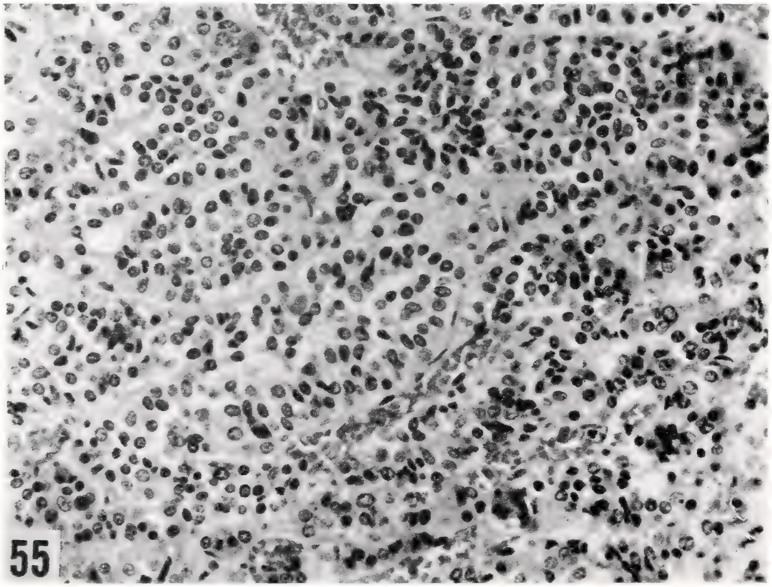
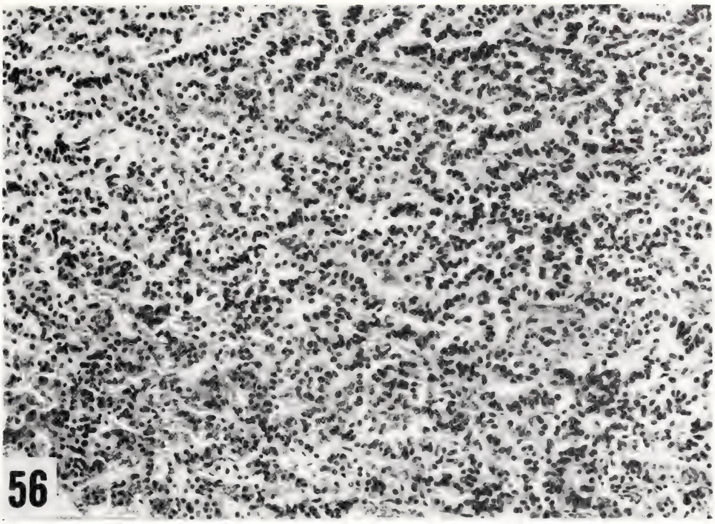


FIGURE 54.—Beta-cell adenoma in pituitary of a 67-year-old woman with atrophic breasts (confirmed by special staining methods).
Hemalum and eosin. × 250. A 9677.

FIGURE 55.—Simple chromophobe adenoma in pituitary of a 31-year-old woman who showed adenosis.
Hemalum and eosin. × 250. B 0057.



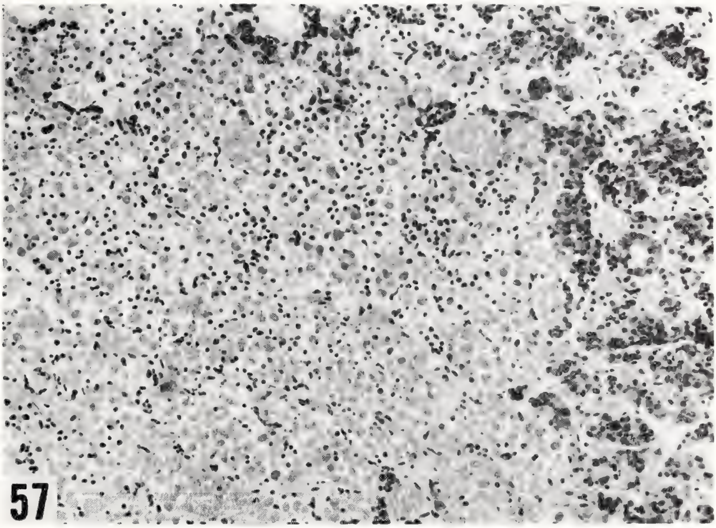
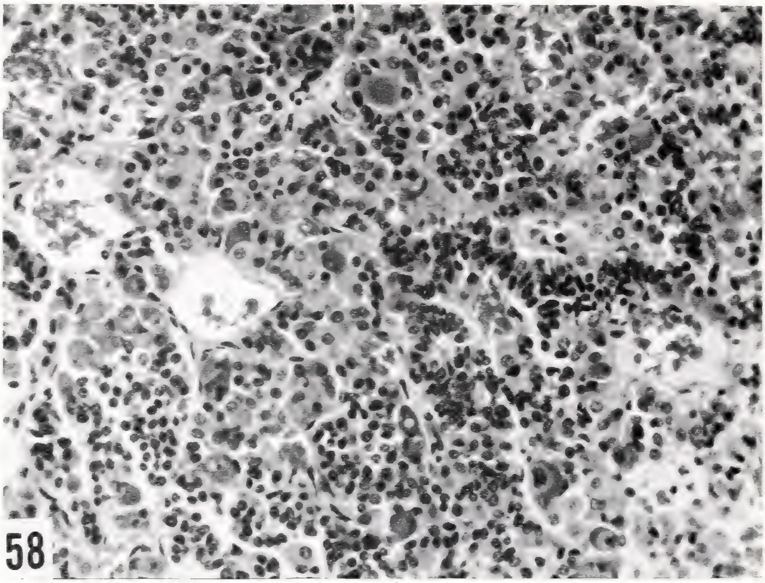


FIGURE 56.—Fetal-cell adenoma of pituitary in a 56-year-old woman who showed adenosis.
Hemalum and eosin. × 130. A 9808.

FIGURE 57.—Patchy acute necrosis of pars anterior of pituitary in a 42-year-old hypertensive woman who showed adenosis.
Hemalum and eosin. × 130. A 9727.



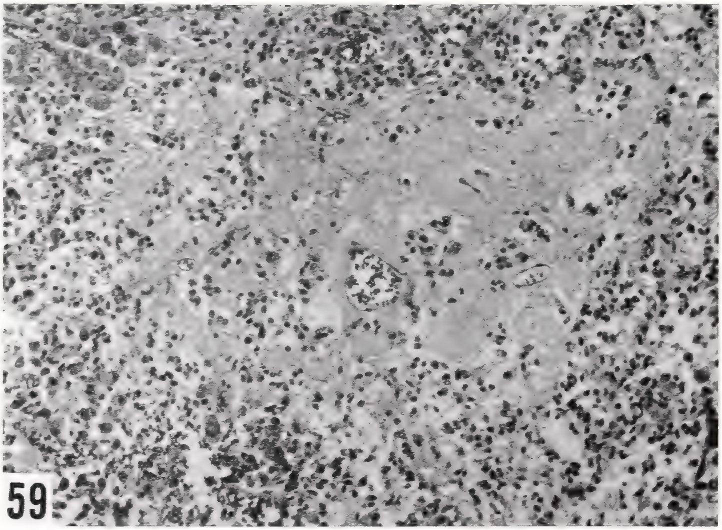
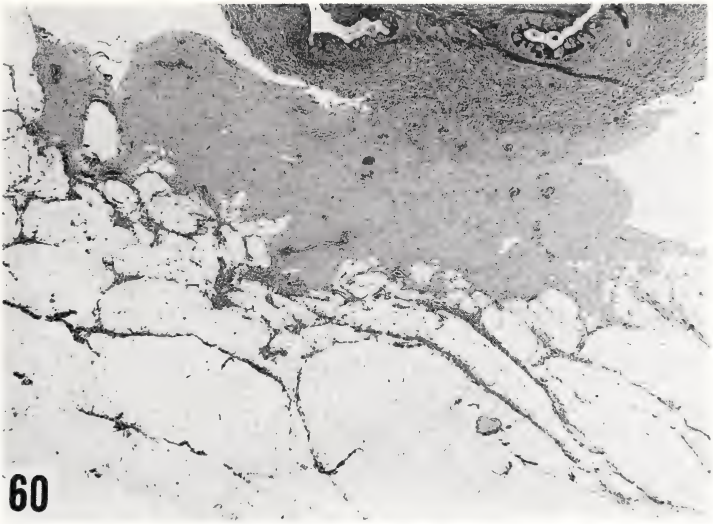


Figure 58.—Fetal chromophobes in pars anterior of pituitary of a 68-year-old woman. Hemalum and eosin. × 250. A 9652.

Figure 59.—Stromal hyalinization of pars anterior of pituitary of a 71-year-old woman who showed epitheliosis.
Hemalum and eosin. × 130. A 9689.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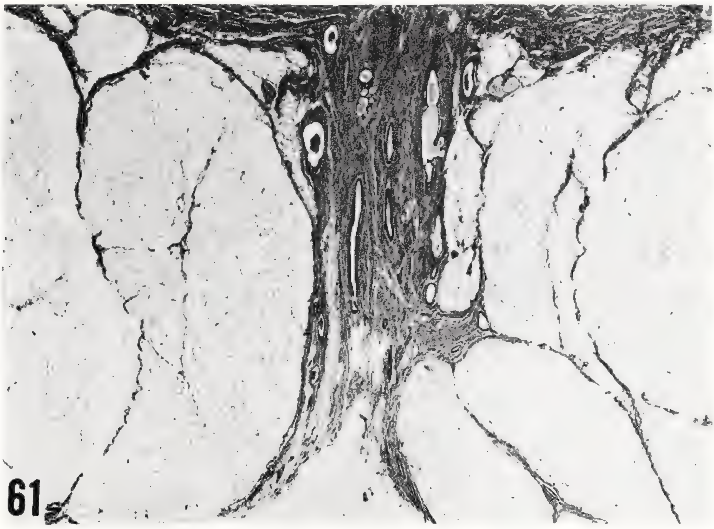
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Figure 60.—Low-power view of whole male breast of normal fibrous type. Hemalum and eosin. × 8. A 8132.

Figure 61.—Low-power view of whole male breast of largely fatty type. Hemalum and eosin. × 8. A 8112.

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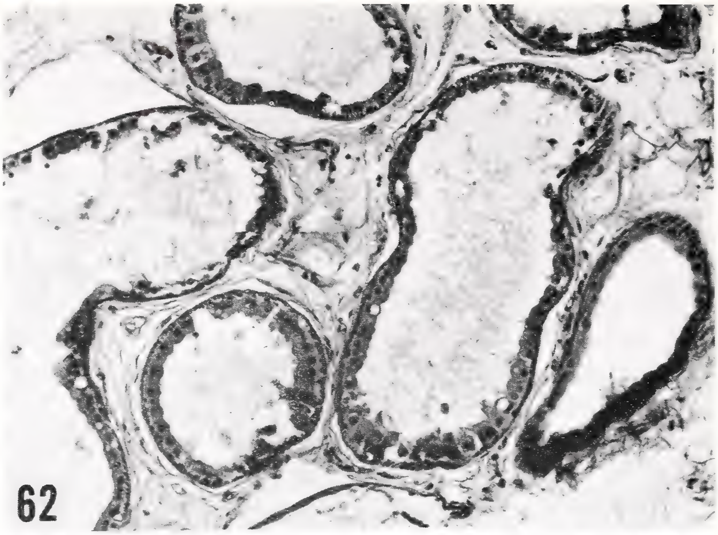


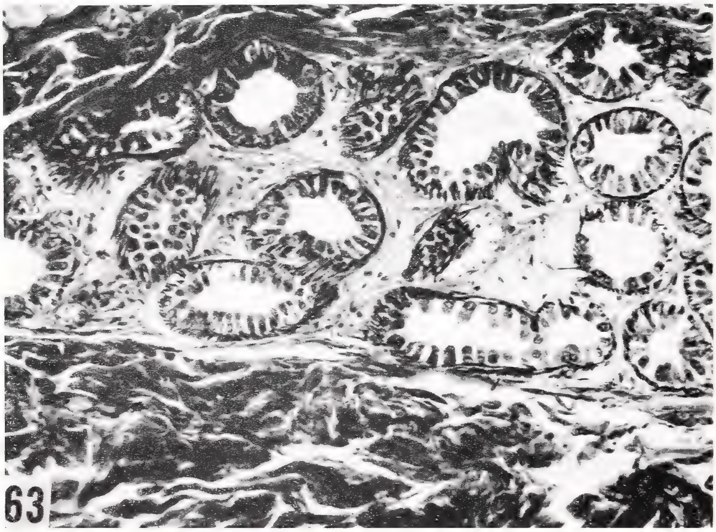
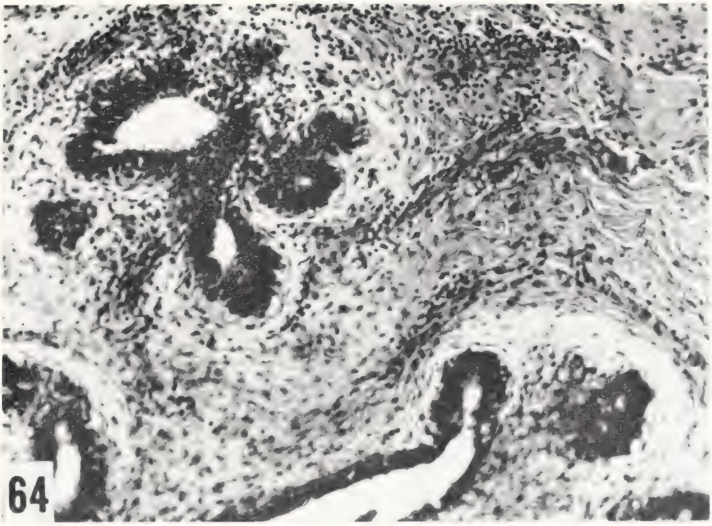


FIGURE 62.—Subareolar apocrine sweat glands in the male. Hemalum and eosin. × 130. A 8040.

FIGURE 63.—Subareolar apocrine sweat glands stained by phosphotungstic acid-hematoxylin to show myoepithelium. × 130. A 8776.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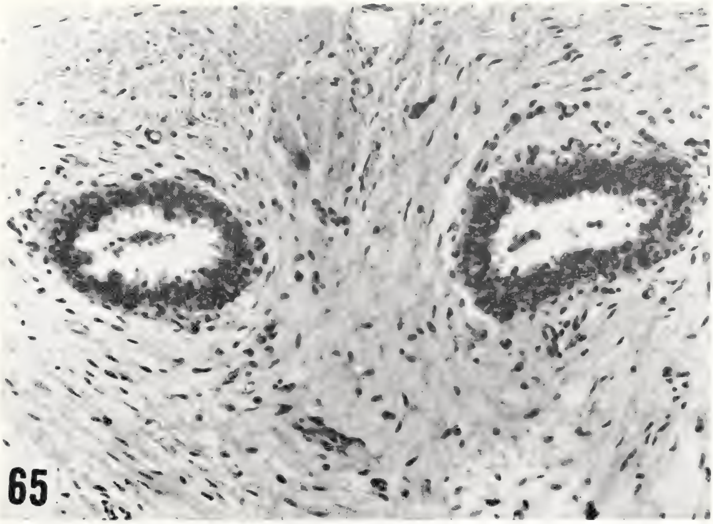
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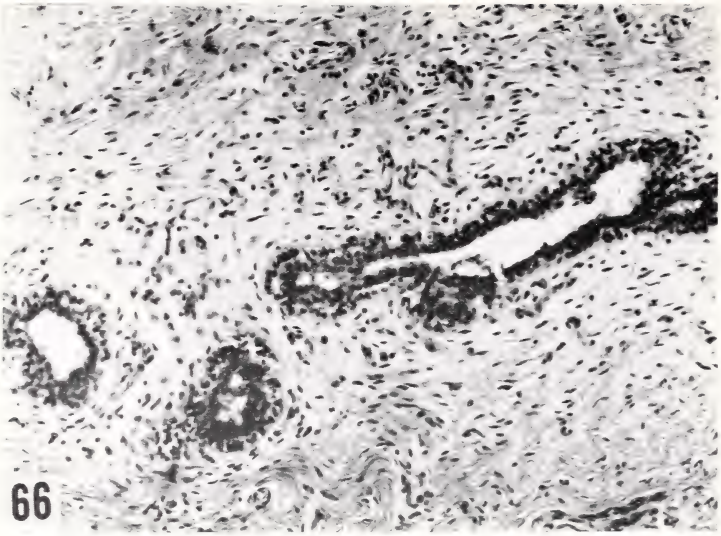
FIGURE 64.—Hypertrophy of breast in a male who died with hepatic carcinomatosis, to show proliferation of epithelium and periductal stroma.
Hemalum and eosin. × 130. A 8472.

FIGURE 65.—Simple hyperplasia of ductal epithelium and formation of fibrillary stroma in a 16-year-old male with Kinnier Wilson's disease.
Hemalum and eosin. × 130. A 8596.

SANDISON



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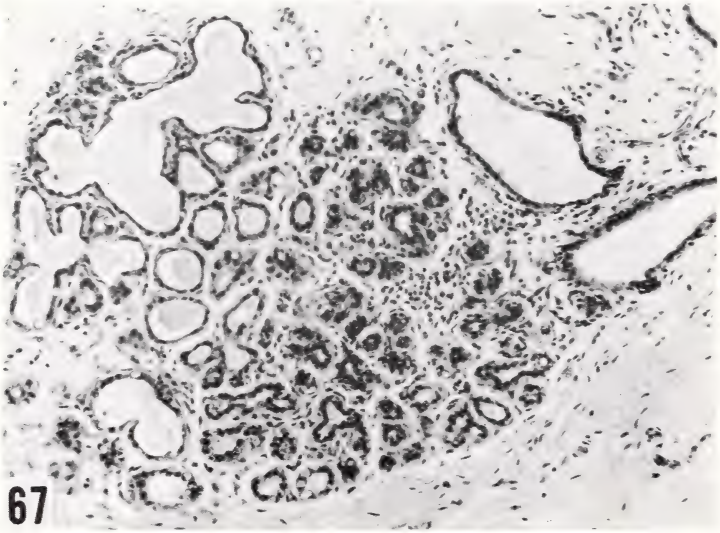

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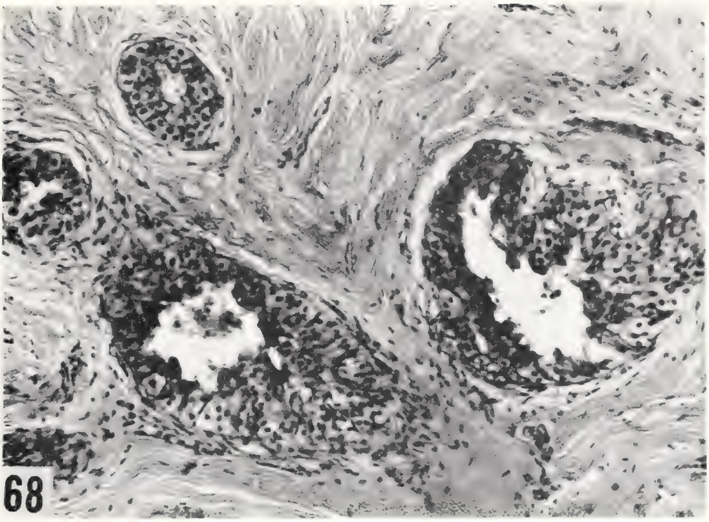
FIGURE 66.—Hypertrophy of male breast involving epithelium and connective-tissue elements after prolonged diethylstilbestrol therapy.
Hemalum and eosin. × 130. A 8948.

FIGURE 67.—Lobule formation after 9 years of diethylstilbestrol therapy for alleged prostatic carcinoma. The only example of lobule formation seen in the male except in chromatin-positive Klinefelter's syndrome.
Hemalum and eosin. × 130. A 9865.
(Courtesy E. and S. Livingstone, Edinburgh)

SANDISON



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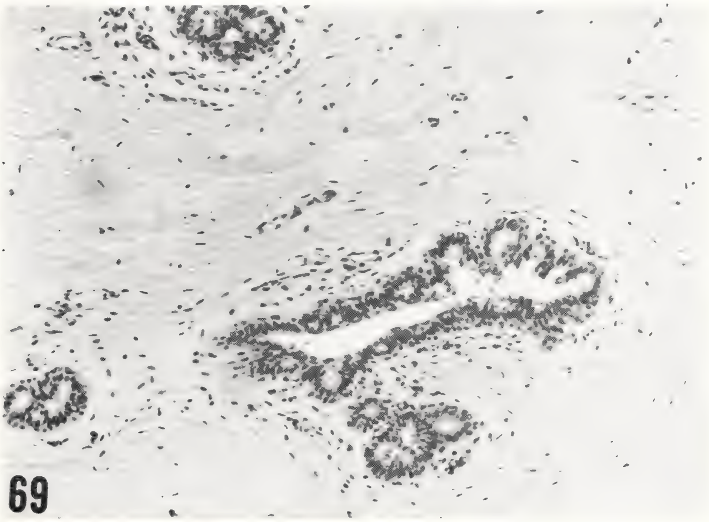

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Figure 68.—Epitheliosis of ducts in a 56-year-old male who died with chronic hepatic venous congestion, the result of aortic stenosis.
Hemalum and eosin. × 130. A 8452.

Figure 69.—Abortive budding of terminal breast ducts in a 61-year-old bronchiectatic male.
Hemalum and eosin. × 130. A 8040.

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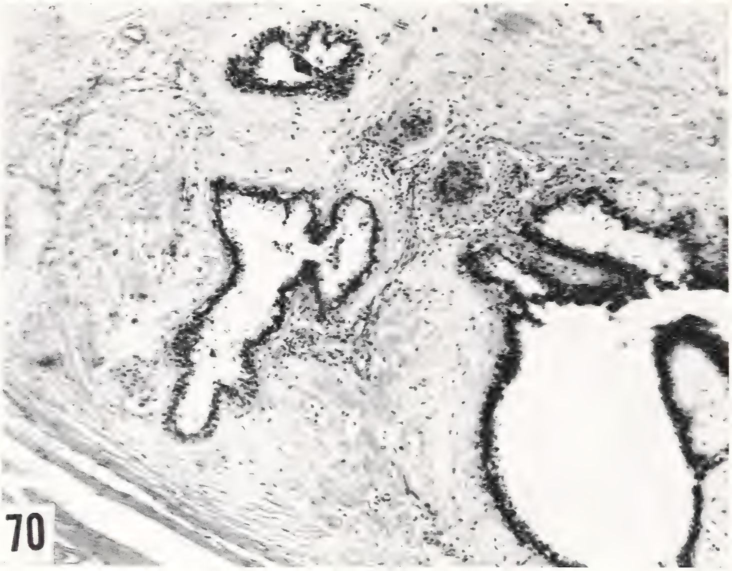

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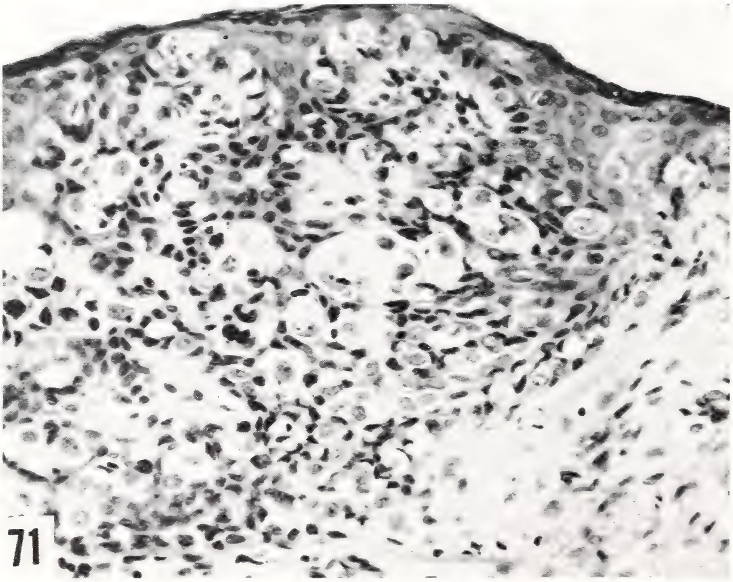

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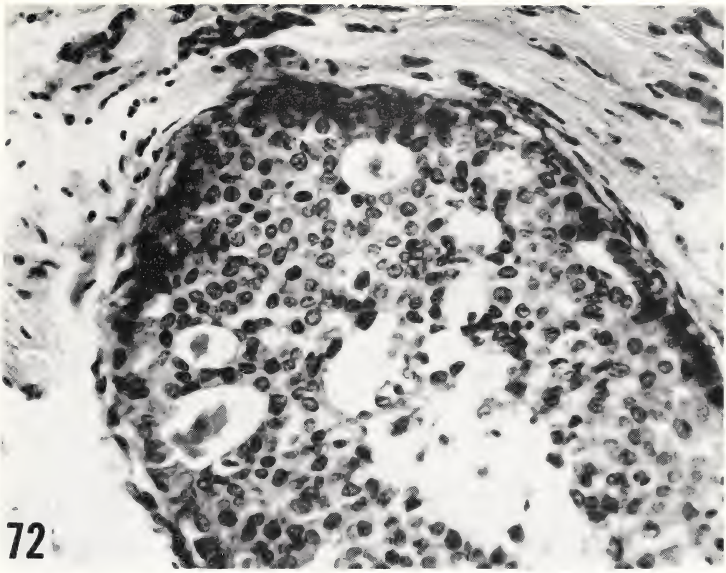
FIGURE 70.—Localized fibroadenomatous area in the breast of a 78-year-old male who died after prostatectomy.
Hemalum and eosin. × 85. A 8036.

FIGURE 71.—Paget's disease of the male breast in a 66-year-old man.
Hemalum and eosin. × 250. A 9264.
(Courtesy British Journal of Surgery)



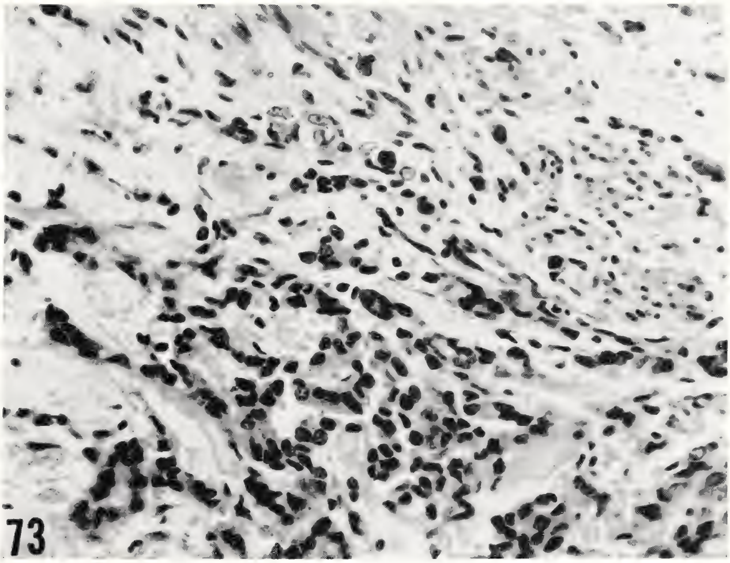
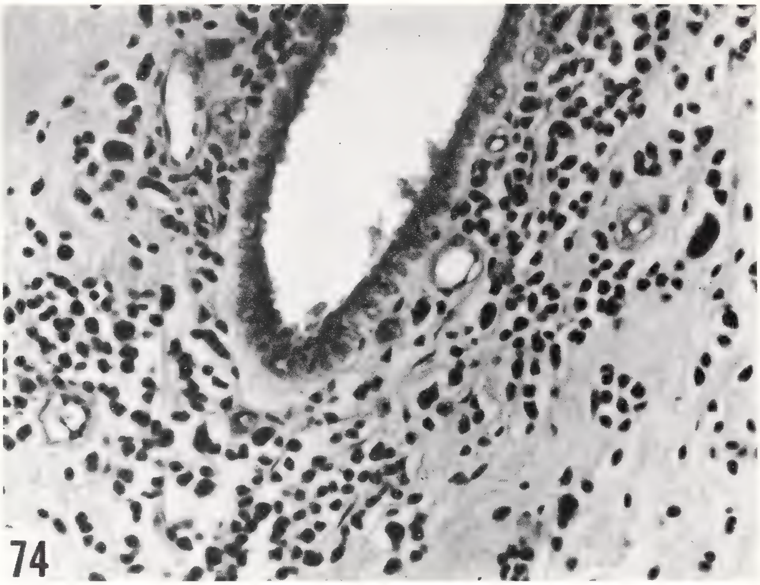


Figure 72.—Intraductal carcinoma in the same case.
Hemalum and eosin. × 250. A 9264.
(Courtesy British Journal of Surgery)

Figure 73.—Infiltrative carcinoma in the same case.
Hemalum and eosin. × 250. A 9264.
(Courtesy British Journal of Surgery)



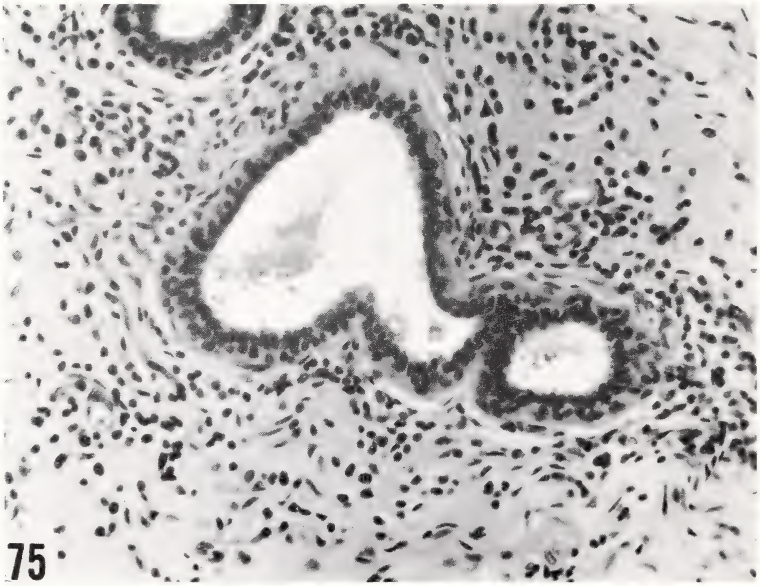


FIGURE 74.—Periductal infiltrate in a 36-year-old male who died of lymphosarcomatosis.
Hemalum and eosin. × 250. A 8608.

FIGURE 75.—Periductal infiltrates in a 31-year-old male who died of eosinophilic leukemia.
Hemalum and eosin. × 250. A 8102.

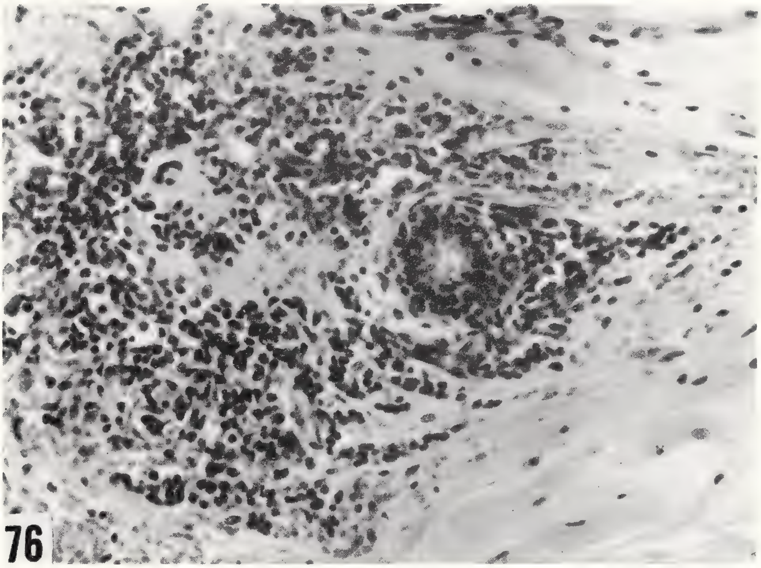


FIGURE 76.—Periductal infiltrates in a 77-year-old male who died of monocytic leukemia.

Hemalum and eosin. × 250. A 8726.

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ADDENDUM

The following list of ages and diagnoses is intended to give some indication of the autopsy population considered in this investigation.

No.	Age	Primary Diagnosis
1	35	Malignant hypertension
2	51	Diabetes mellitus
3	73	Carcinoma, pancreas
4	30	Simmonds' disease
5	33	Appendicitis; pulmonary embolism
6	62	Bladder, sarcoma
7	72	Bronchial carcinoma
8	74	Perforated peptic ulcer
9	28	Malignant hypertension
10	75	Carcinoma, breast
11	44	Barbiturate poisoning
12	55	Subarachnoid hemorrhage
13	71	Cervical cord softening
14	71	Hypertension
15	46	Perforated peptic ulcer
16	62	Coronary thrombosis
17	57	Cerebral abscess
18	71	Carcinoma, kidney
19	71	Hypertension
20	86	Fractured femur
21	67	Suppurative cholangitis
22	44	Chronic myeloid leukemia
23	60	Bronchial carcinoma
24	70	Fractured skull
25	63	Osteomalacia; congestive failure
36	67	Diverticulitis
27	73	Carcinoma, stomach
28	55	Chronic glomerulonephritis
29	54	Carcinoma, stomach
30	50	Chronic pyelonephritis
31	67	Coronary occlusion
32	57	Hernia repair: bronchopneumonia
33	51	Chronic myeloid leukemia
34	37	Cerebral hemorrhage
35	36	Gastroenterostomy: pulmonary embolism
36	55	Lymphosarcoma
37	59	Acute pancreatitis
38	51	Diabetes mellitus
39	72	Subarachnoid hemorrhage
40	71	Carcinoma, colon
41	68	Carcinoma, bile duct
42	65	Appendicitis: phlebothrombosis

No.	Age	Primary Diagnosis
43	56	Coronary thrombosis
44	66	Hypertension
45	76	Cavernous sinus thrombosis
46	67	Bronchopneumonia
47	64	Carcinoma, stomach
48	68	Peritonitis
49	59	Carcinoma, colon
50	76	Pituitary giant-cell granuloma
51	31	Acute appendicitis
52	50	Carcinoma, stomach
53	41	Peritonitis: liver necrosis
54	70	Myocardial infarction
55	72	Septicemia: cellulitis
56	45	Mitral stenosis
57	66	Carcinoma, colon
58	52	Hypertension
59	33	Carcinoma, stomach
60	64	Tuberculous osteitis
61	67	Aortic stenosis
62	50	Cerebral hemorrhage
63	36	Coronary occlusion
64	62	Bronchial carcinoma
65	49	Bronchial carcinoma
66	48	Carcinoma, breast
67	53	Hepatic cirrhosis
68	63	Astrocytoma
69	51	Bronchial carcinoma
70	61	Diabetes mellitus
71	60	Polyarteritis nodosa
72	72	Coronary thrombosis
73	39	Bronchial carcinoma
74	70	Thyroid adenoma
75	54	Subarachnoid hemorrhage
76	56	Status asthmaticus
77	50	Bronchiectasis
78	49	Polioencephalitis
79	31	Hypertension
80	37	Mitral stenosis
81	31	Diabetes mellitus
82	68	Femoral embolectomy
83	50	Carcinoma, colon
84	55	Chronic glomerulonephritis
85	61	Mitral stenosis
86	46	Femoral vein thrombosis
87	56	Carcinoma, breast
88	60	Myxoedema
89	72	Cerebral hemorrhage
90	64	Chronic bronchitis
91	67	Femoral embolism
92	30	Hodgkin's disease
93	51	Bronchial carcinoma
94	75	Subarachnoid hemorrhage
95	29	Addison's disease
96	76	Hepatic cirrhosis
97	74	Diabetes mellitus

No.	Age	Primary Diagnosis
98	45	Mitral stenosis
99	56	Melanoma, breast
100	69	Bronchial carcinoma
101	63	Diabetes mellitus
102	55	Acute meningitis
103	62	Diverticulitis
104	29	Mitral stenosis
105	41	Cerebral hemorrhage
106	62	Congestive failure
107	46	Steatorrhea
108	62	Thyrotoxicosis
109	80	Carcinoma, colon
110	63	Chronic bronchitis
111	67	Carcinoma, breast
112	72	Carcinoma, ovary
113	59	Cerebral hemorrhage
114	77	Pulmonary embolus
115	68	Acute iodine poisoning
116	56	Ileal lymphosarcoma
117	57	Polyarteritis nodosa
118	39	Multiple fractures
119	71	Chronic bronchitis
120	53	Subacute liver necrosis
121	57	Diabetes mellitus
122	56	Mesenteric artery thrombosis
123	74	Diabetes mellitus
124	53	Hypertension
125	71	Ruptured aortic aneurysm
126	73	Diabetes mellitus
127	60	Chronic pyelonephritis
128	43	Chronic bronchitis
129	55	Pyopneumothorax
130	65	Subarachnoid hemorrhage: carcinoma, breast
131	71	Aortic stenosis
132	61	Pyonephrosis
133	61	Carcinoma, stomach
134	62	Carcinoma, stomach
135	61	Diabetes mellitus
136	72	Coronary thrombosis
137	53	Chronic bronchitis
138	49	Chronic glomerulonephritis
139	60	Fractured skull
140	60	Subarachnoid hemorrhage
141	60	Myxoedema
142	64	Carcinoma, bile duct
143	38	Chronic glomerulonephritis
144	66	Obstructive jaundice
145	60	Carcinoma, bladder
146	47	Bronchiectasis
147	71	Anemia
148	55	Acute leukemia
149	63	Myocardial infarction
150	55	Mitral stenosis
151	70	Myxoedema
152	66	Cholelithiasis

No.	Age	Primary Diagnosis
153	70	Carcinoma, stomach
154	63	Bronchial carcinoma
155	64	Carcinoma, breast
156	66	Carcinoma, pancreas
157	72	Carcinoma, rectum
158	36	Mitral stenosis
159	73	Ileal carcinoid
160	24	Subacute glomerulonephritis
161	63	Carcinoma, breast
162	53	Pyelonephritis
163	59	Cerebral hemorrhage
164	49	Aortic stenosis
165	75	Bronchial carcinoma
166	71	Cerebral hemorrhage
167	79	Cerebral thrombosis
168	52	"Persomnia" poisoning
169	44	Encephalomyelitis
170	23	Aortic stenosis
171	70	Thyrotoxicosis
172	60	Portal thrombosis
173	56	Carcinoma, breast
174	45	Carcinoma, esophagus
175	58	Carcinoma, gallbladder
176	66	Carcinoma, colon
177	72	Bronchial carcinoma
178	55	Hepatic postnecrotic scarring
179	68	Bronchial carcinoma
180	45	Thrombocytopenic purpura
181	48	Mitral stenosis
182	72	Carcinoma, rectum
183	36	Mitral stenosis
184	67	Cholangitis
185	55	Acute cresol poisoning
186	33	Regional ileitis
187	54	Carcinoma, ovary
188	77	Fractured femur
189	66	Coronary thrombosis
190	52	Myocardial infarction
191	40	Biliary cirrhosis
192	81	Thyrotoxicosis
193	68	Diabetes mellitus
194	61	Bronchial carcinoma
195	55	Cholelithiasis
196	42	Myelomatosis
197	66	Fractured skull
198	65	Carcinoma, rectum
199	31	Chronic glomerulonephritis
200	48	Post-hysterectomy ileus
201	57	Myocardial infarction
202	54	Bronchial carcinoma
203	62	Carcinoma, colon
204	35	Mitral stenosis
205	73	Pyelonephritis
206	66	Pyonephrosis
207	61	Hepatic nodular hyperplasia

No.	Age	Primary Diagnosis
208	64	Hypertension
209	77	Carcinoma, gallbladder
210	47	Cerebral hemorrhage
211	78	Chronic gastric ulcer
212	22	Pulmonary tuberculosis
213	65	Myomalacia cordis
214	65	Diverticulitis
215	78	Cholecystitis: peritonitis
216	49	Malignant hypertension
217	71	Tuberculous peritonitis
218	62	Aortic incompetence
219	60	Ulcerative colitis
220	46	Bronchial carcinoma
221	73	Coronary occlusion
222	64	Carcinoma, esophagus
223	56	Bleeding peptic ulcer
224	68	Hepatic cirrhosis
225	40	Cerebral hemorrhage
226	75	Acute intestinal obstruction
227	67	Coronary occlusion
228	80	Peritonitis: pulmonary embolus
229	47	Pulmonary tuberculosis
230	60	Cystitis
231	34	Ulcerative colitis
232	64	Diabetes mellitus
233	46	Lobar pneumonia
234	70	Chronic bronchitis
235	72	Burns
236	32	Hypertension
237	86	Carcinoma, colon
238	22	Mitral stenosis
239	51	Cholecystitis
240	48	Myocardial infarction
241	31	Subacute glomerulonephritis
242	65	Hydronephrosis
243	68	Intestinal ileus
244	65	Hepatic cirrhosis
245	32	Chorionepithelioma
246	58	Coronary thrombosis
247	69	Pyelonephritis
248	65	Fractured skull
249	35	Carcinoma, colon
250	57	Myomalacia cordis
251	67	Carcinoma, breast
252	58	Carcinoma, colon
253	56	Carcinoma, stomach
254	46	Mesenteric tuberculosis
255	67	Portal thrombosis
256	44	Appendicitis: peritonitis
257	51	Coronary thrombosis
258	40	Mitral stenosis
259	63	Strangulated hernia
260	61	Fractured femur
261	76	Peptic ulcer perforation
262	56	Hodgkin's disease

No.	Age	Primary Diagnosis
263	56	Acute tracheobronchitis
264	58	Pulmonary embolus
265	31	Mitral stenosis
266	53	Acute bronchitis
267	68	Diabetes mellitus
268	84	Achalasia, cardia
269	55	Hepatic cirrhosis
270	72	Carcinoma, stomach
271	32	Sagittal sinus thrombosis
272	59	Mesenteric embolus
273	68	Diverticulitis
274	54	Carcinoma, bladder
275	60	Status epilepticus
276	73	Myocardial infarction
277	54	Thyrotoxicosis
278	61	Myocardial infarction
279	72	Pontine softening
280	68	Bronchopneumonia
281	76	Aortic stenosis
282	84	Pulmonary tuberculosis
283	50	Cerebral softening
284	61	Acute meningitis
285	70	Osteosarcoma, femur
286	64	Pyelonephritis
287	53	Carcinoma, ovary
288	55	Reticulosarcoma
289	69	Lobar pneumonia
290	47	Pyelonephritis
291	67	Acute tracheobronchitis
292	31	Diabetes mellitus
293	61	Carcinoma, pancreas
294	80	Diverticulitis
295	72	Bronchopneumonia
296	79	Aortic aneurysm
297	64	Pulmonary embolism
298	68	Pontine hemorrhage
299	42	Mitral stenosis
300	61	Cholangiocarcinoma
301	31	Diabetes mellitus
302	73	Cerebellar hemorrhage
303	49	Malignant hypertension
304	42	Mitral stenosis
305	79	Carcinoma, bile duct
306	63	Carcinoma, breast
307	65	Perforated peptic ulcer
308	71	Fractured spine
309	72	Ruptured aorta
310	49	Subarachnoid hemorrhage
311	78	Perforation, rectum
312	72	Carcinoma, stomach
313	23	Mitral stenosis
314	62	Mycosis fungoides
315	31	Appendicitis
316	60	Coronary thrombosis
317	71	Coronary thrombosis

No.	Age	Primary Diagnosis
318	34	Subacute glomerulonephritis
319	66	Portal thrombosis
320	54	Mitral stenosis
321	66	Diverticulitis
322	64	Hepatic cirrhosis
323	46	Pituitary necrosis
324	57	Diabetes mellitus
325	53	Addison's disease
326	68	Cerebral hemorrhage
327	40	Acute liver necrosis
328	63	Mitral stenosis
329	64	Carcinoma, breast
330	70	Carcinoma, colon
331	47	Chronic peptic ulcer
332	51	Hepatic cirrhosis
333	47	Mitral stenosis
334	44	Carcinoma, breast
335	69	Hepatic cirrhosis
336	54	Aortic stenosis
337	29	Acute poisoning
338	54	Disseminated tuberculosis
339	52	Coronary thrombosis
340	52	Oligodendroglioma
341	55	Myocardial infarction
342	74	Intestinal obstruction
343	32	Mitral stenosis
344	69	Biliary cirrhosis
345	66	Appendicectomy: pulmonary embolus
346	78	Hepatic cirrhosis
347	46	Oophorectomy: ileus
348	70	Cardiac arrhythmia
349	62	Hepatic cirrhosis
350	66	Cerebral thrombosis
351	57	Carcinoma, breast
352	73	Myocardial infarction
353	60	Inanition
354	33	Chronic glomerulonephritis
355	63	Simmonds' disease
356	53	Hepatic cirrhosis
357	76	Pontine hemorrhage
358	70	Cerebral hemorrhage
359	69	Carcinoma, jejunum
360	43	Pyosalpinx
361	63	Chronic pyelonephritis
362	63	Cerebral hemorrhage
363	78	Fractured femur
364	54	Carcinoma, stomach
365	65	Myxoedema: diabetes mellitus
366	64	Carcinoma, colon
367	42	Carcinoma, uterus
368	33	Aortic stenosis
369	71	Mitral stenosis
370	73	Subarachnoid hemorrhage
371	65	Carcinoma, pancreas
372	54	Subarachnoid hemorrhage

No.	Age	Primary Diagnosis
373	31	Mitral stenosis
374	76	Tumor, ampulla of Vater
375	58	Myocardial infarction
376	60	Diabetes mellitus
377	47	Mitral stenosis
378	58	Coronary thrombosis
379	45	Hepatic cirrhosis
380	64	Monocytic leukemia
381	68	Carcinoma, colon
382	68	Carcinoma, breast
383	65	Hypertension
384	56	Pulmonary tuberculosis
385	69	Strangulated hernia
386	62	Bronchial carcinoma
387	59	Mitral stenosis
388	56	Chronic bronchitis
389	45	Chronic bronchitis
390	46	Chronic glomerulonephritis
391	67	Chronic pyelonephritis
392	54	Mitral stenosis
393	76	Cerebral lacerations
394	71	Coronary thrombosis: diabetes mellitus
395	53	Chronic glomerulonephritis
396	65	Coronary thrombosis
397	65	Lobar pneumonia
398	61	Thyroid lymphosarcoma
399	79	Coronary thrombosis
400	64	Acute nephritis
401	60	Chronic myeloid leukemia
402	72	Chronic peptic ulcer
403	63	Pernicious anemia
404	52	Chronic peptic ulcer
405	45	Senear-Usher syndrome
406	71	Carcinoma, bile duct
407	55	Pyonephrosis
408	63	Chronic peptic ulcer
409	63	Mitral stenosis
410	40	Mitral stenosis
411	40	Polycystic kidneys
412	61	Mitral stenosis
413	55	Bronchial carcinoma
414	76	Coronary thrombosis
415	52	Ulcerative colitis
416	54	Pemphigus foliaceus
417	35	Bronchial asthma
418	65	Coronary thrombosis
419	70	Hypertension
420	60	Bronchial carcinoma
421	75	Ileal infarction
422	73	Post-hernial repair
423	45	Hepatic cirrhosis
424	61	Malignant granulosa-cell tumor
425	74	Acute hemorrhagic pancreatitis
426	55	Hemochromatosis
427	78	Chronic bronchitis

No.	Age	Primary Diagnosis
428	58	Wernicke's encephalopathy
429	69	Carcinoma, pancreas
430	75	Multiple peptic ulcers
431	23	Volvulus, colon
432	75	Subarachnoid hemorrhage
433	66	Coronary thrombosis
434	59	Chronic peptic ulcer
435	67	Hypertension
436	49	Cholecystectomy
437	65	Carcinoma, gallbladder
438	44	Mitral stenosis
439	38	Mitral stenosis
440	40	Malignant astrocystoma
441	49	Mitral stenosis
442	54	Malignant hypertension
443	76	Cerebral softening
444	67	Chronic bronchitis
445	40	Subarachnoid hemorrhage
446	65	Cholecystectomy
447	69	Carcinoma, stomach
448	56	Cholecystectomy
449	38	Bronchial asthma
450	61	Carcinoma, stomach
451	47	Mitral stenosis
452	74	Strangulated hernia
453	65	Carcinoma, ovary
454	58	Status asthmaticus
455	54	Carcinoma, pancreas
456	48	Mitral stenosis
457	62	Rheumatoid arthritis
458	50	Chronic pyelonephritis
459	52	Coronary thrombosis
460	62	Coronary thrombosis
461	55 (*circa*)	Subarachnoid hemorrhage
462	65	Strangulated hernia
463	64	Cerebral laceration
464	64	Hypertension
465	49	Post-necrotic liver scarring
466	43	Diabetes mellitus
467	83	Ileal infarction
468	37	Progressive glomerulonephritis
469	56	Chronic glomerulonephritis
470	48	Hysterectomy: pulmonary embolus
471	32	Mitral stenosis
472	41	Mitral stenosis
473	63	Carcinoma, bladder
474	75	Myocardial fibrosis
475	81	Cerebral hemorrhage
476	36	Disseminated lupus erythematosus
477	72	Diabetes mellitus
478	62	Diabetes mellitus
479	59	Carcinoma, ampulla of Vater
480	64	Chronic pyelonephritis
481	69	Duodenal obstruction
482	42	Mitral stenosis

No.	Age	Primary Diagnosis
483	86	Calcific aortic stenosis
484	64	Mitral stenosis
485	63	Diabetes mellitus
486	66	Cholangitis
487	42	Mitral stenosis
488	53	Mitral stenosis
489	48	Portal thrombosis
490	51	Reticulosarcoma
491	43	Pulmonary tuberculosis
492	60	Hypertension
493	73	Bronchial carcinoma
494	62	Cholecystectomy
495	65	Coronary thrombosis
496	68	Coronary thrombosis
497	48	Chronic glomerulonephritis
498	72	Coronary thrombosis
499	63	Giant-cell tumor, bone
500	46	Melancholia: inanition
501	49	Carcinoma, nose
502	72	Subarachnoid hemorrhage
503	74	Chronic bronchitis
504	65	Malignant myoblastoma
505	62	Carcinoma, bladder
506	69	Mycosis fungoides
507	65	Subarachnoid hemorrhage
508	52	Phlebothrombosis: pulmonary embolus
509	62	Carcinoma, renal pelvis
510	66	Oophorectomy: pulmonary embolus
511	80	Aortic thrombosis
512	69	Mesenteric artery thrombosis
513	60	Mitral stenosis
514	62	Pheochromocytoma
515	25	Cerebral abscess
516	50	Cystadenoma, ovary
517	71	Bronchial carcinoma
518	58	Coronary occlusion
519	59	Coronary thrombosis
520	21	Status asthmaticus
521	34	Microangiopathic thrombotic purpura
522	30	Criminal abortion
523	69	Anesthetic death
524	51	Disseminated sclerosis
525	54	Carcinoma, breast
526	54	Coronary thrombosis
527	52	Chronic peptic ulcer
528	78	Coronary thrombosis
529	65	Carcinoma, cervix uteri
530	43	Encephalitis
531	35	Chronic pyelonephritis
532	73	Diverticulitis
533	22	Acute rheumatic carditis
534	66	Carcinoma, colon
535	68	Bronchial carcinoma
536	62	Cholecystectomy
537	55	Carcinoma, colon

No.	Age	Primary Diagnosis
538	60	Chronic bronchitis
539	54	Coronary thrombosis
540	51	Ulcerative colitis
541	65	Cerebral softening
542	62	Cerebral hemorrhage
543	64	Hypertension
544	70	Cerebral hemorrhage
545	54	Myelofibrosis
546	55	Bronchial carcinoma
547	73	Myocardial fibrosis
548	55	Mitral stenosis
549	32	Cerebral softening
550	60	Thyrotoxicosis
551	63	Chronic peptic ulcer
552	70	Cerebral hemorrhage
553	51	Cholecystectomy
554	54	Pelvic sarcoma
555	60	Cerebral hemorrhage
556	28	Anesthetic death
557	73	Coronary thrombosis
558	73	Myocardial infarction
559	47	Malignant astrocytoma
560	53	Mitral stenosis
561	34	Mitral stenosis
562	62	Marrow hypoplasia
563	65	Chronic pyelonephritis
564	59	Myomalacia cordis
565	62	Cerebral softening
566	55	Subarachnoid hemorrhage
567	54	Cavernous transformation portal vein
568	69	Carcinoma, rectum
569	40	Diabetes mellitus
570	22	Chronic pyelonephritis
571	63	Myxoedema
572	47	Diabetes mellitus
573	70	Empyema gallbladder
574	70	Coronary thrombosis
575	62	Myocardial fibrosis
576	78	Fractured femur
577	82	Fractured femur
578	69	Stomal ulcer
579	53	Chronic bronchitis
580	63	Chronic bronchitis
581	40	Carcinoma, breast
582	65	Diabetes mellitus
583	33	Cerebral hemorrhage
584	70	Chronic bronchitis
585	69	Carcinoma, breast
586	69	Carcinoma, ovary
587	51	Subarachnoid hemorrhage
588	85	Cerebral hemorrhage
589	47	Cerebral softening
590	51	Coronary thrombosis
591	36	Diabetes mellitus
592	48	Amytal poisoning

No.	Age	Primary Diagnosis
593	52	Hepatic cirrhosis
594	31	Barbiturate poisoning
595	30	Carcinoma, breast
596	60	Coronary thrombosis
597	82	Carcinoma, stomach
598	53	Rheumatoid arthritis
599	57	Diabetes mellitus
600	42	Dysgerminoma, ovary
601	60	Subarachnoid hemorrhage
602	56	Carcinoma, bladder
603	84	Fractured femur
604	62	Fractured skull
605	66	Diabetes mellitus
606	68	Chronic pyelonephritis
607	72	Myocardial fibrosis
608	67	Diabetes mellitus
609	40	Carcinoma, breast
610	60	Myocardial fibrosis
611	69	Pernicious anemia
612	62	Pulmonary tuberculosis
613	68	Myocardial infarction
614	78	Chronic pyelonephritis
615	55	Coronary thrombosis
616	62	Diabetes mellitus
617	59	Diabetes mellitus
618	52	Barbiturate poisoning
619	67	Perforated peptic ulcer
620	63	Acute bronchitis
621	42	Myocardial infarction
622	64	Acute hemorrhagic pancreatitis
623	64	Coronary occlusion
624	71	Diabetes mellitus: tuberculosis
625	80	Myxoedema
626	60	Acute hepatic necrosis
627	88	Pernicious anemia
628	32	Mitral stenosis
629	66	Carcinoma, ovary
630	54	Subarachnoid hemorrhage
631	50	Hysterectomy: pulmonary embolus
632	77	Calcific aortic stenosis
633	68	Achalasia, esophagus
634	63	Carcinoma, pancreas
635	31	Carcinoma, breast
636	59	Carcinoma, breast
637	44	Cerebral hemorrhage
638	42	Hypertension
639	55	Myocardial fibrosis
640	60	Coronary thrombosis
641	43	Mitral stenosis
642	29	Mitral stenosis
643	72	Carcinoma, gallbladder
644	62	Chronic pyelonephritis
645	75	Diverticulitis
646	74	Chronic bronchitis
647	42	Carcinoma, breast

No.	Age	Primary Diagnosis
648	75	Carcinoma, stomach
649	73	Carcinoma, pancreas
650	58	Carcinoma, breast
651	59	Myocardial infarction
652	52	Pentobarbitone (pentobarbital) poisoning
653	71	Peptic ulcer
654	62	Post-herniorrhaphy death
655	70	Mitral stenosis
656	69	Polyarteritis nodosa
657	75	Pyelonephritis
658	60	Pernicious anemia
659	51	Chronic pyelonephritis
660	55	Malignant astrocytoma
661	68	Bronchial carcinoma
662	65	Carcinoma, corpus uteri
663	52	Cholelithiasis
664	66	Carcinoma, stomach
665	42	Ulcerative colitis
666	56	Cerebral hemorrhage
667	54	Cerebral hemorrhage
668	42	Malignant astrocytoma
669	60	Mitral stenosis
670	65	Carcinoma, vulva
671	66	Chronic glomerulonephritis
672	64	Acute pyelonephritis
673	57	Chronic pyelonephritis
674	62	Coronary thrombosis
675	46	Cerebral hemorrhage
676	21	Diabetes mellitus
677	39	Barbiturate poisoning
678	67	Coronary occlusion
679	76	Carcinoma, stomach
680	46	Carcinoma, rectum
681	47	Polycystic kidneys
682	57	Cerebral abscess
683	59	Hypertension
684	50	Chronic pyelonephritis
685	45	Chronic pyelonephritis
686	74	Pernicious anemia
687	43	Chronic pyelonephritis
688	67	Diabetes mellitus
689	70	Fractured femur
690	60	Cerebral hemorrhage
691	63	Mitral stenosis
692	60	Cerebral hemorrhage
693	58	Acute pancreatitis
694	29	Thyrotoxicosis
695	48	Cerebellar hemorrhage
696	73	Chronic pyelonephritis
697	48	Carcinoma, breast
698	62	Dissecting aortic aneurysm
699	71	Cholangitis
700	69	Polyarteritis nodosa
701	64	Malignant astrocytoma
702	41	Miliary tuberculosis
703	59	Subarachnoid hemorrhage

No.	Age	Primary Diagnosis
704	52	Bronchial asthma
705	81	Carcinoma, colon
706	40	Carcinoma, breast
707	50	Bronchial carcinoma
708	79	Subacute peptic ulcer
709	33	Accidental hemorrhage at term
710	84	Carcinoma, breast
711	55	Carcinoma, breast
712	33	Carcinoma, breast
713	72	Acute ileocolitis
714	32	Malignant hypertension
715	43	Carcinoma, breast
716	45	Malignant hypertension
717	77	Cerebral hemorrhage
718	67	Diverticulitis
719	59	Carcinoma, rectum
720	58	Diabetes mellitus
721	50	Mitral stenosis
722	75	Gastric papilloma
723	58	Cerebral softening
724	60	Acute histiocytic medullary reticulosis
725	51	Mitral stenosis
726	71	Carcinoma, pancreas and breast
727	48	Malignant lymphoid neoplasm
728	53	Malignant hypertension
729	69	Carcinoma, jejunum
730	68	Strangulated hernia
731	51	Mitral stenosis
732	68	Ocular melanoma
733	76	Peptic ulcer
734	30	Chronic glomerulonephritis
735	75	Fractures, pelvis and humerus
736	68	Myocardial infarction
737	69	Phlebothrombosis: pulmonary embolus
738	53	Addison's disease
739	60	Subarachnoid hemorrhage
740	55	Carcinoma, rectum
741	31	Hodgkin's disease
742	76	Cerebral hemorrhage
743	65	Subacute glomerulonephritis
744	33	Appendicectomy: peritonitis
745	35	Chronic pyelonephritis
746	70	Coronary thrombosis
747	69	Carcinoma, breast
748	58	Carinoma, breast
749	71	Coronary thrombosis
750	52	Recent herniorrhaphy
751	75	Bronchial carcinoma
752	42	Esophageal perforation
753	23	Diabetes mellitus
754	65	Coronary thrombosis
755	71	Cholangitis
756	65	Myocardial infarction
757	57	Mitral stenosis
758	34	Mitral stenosis

No.	Age	Primary Diagnosis
759	71	Carcinoma, breast
760	73	Myxoedema
761	64	Bronchiectasis
762	75	Carcinoma, colon
763	62	Chronic bronchitis
764	77	Chronic peptic ulcer
765	24	Mitral stenosis
766	69	Cerebral softening
767	88	Phlebothrombosis: pulmonary embolus
768	75	Myocardial infarction
769	61	Cor pulmonale
770	64	Carcinoma, ovary
771	65	Perforation, bowel
772	74	Cerebral hemorrhage
773	38	Disseminated sclerosis
774	64	Carcinoma, breast
775	70	Lobar pneumonia
776	69	Coronary thrombosis
777	41	Carcinoma, ovary
778	71	Colitis
779	33	Malignant melanoma
780	61	Carcinoma, breast
781	57	Myocardial infarction
782	64	Myocardial infarction
783	40	Carcinoma, cervix uteri
784	66	Carcinoma, corpus uteri
785	39	Myomectomy: pulmonary embolus
786	32	Pulmonary tuberculosis
787	32	Ulcerative colitis
788	36	Chiari's syndrome
789	67	Carcinoma, stomach
790	71	Macrocytic anemia
791	83	Lobar pneumonia
792	45	Subacute glomerulonephritis
793	68	Coronary thrombosis
794	44	Pontine hemorrhage
795	63	Acute encephalitis
796	65	Cerebral softening
797	66	Carcinoma, breast
798	54	Continuing hepatitis
799	40	Mitral stenosis
800	74	Chronic peptic ulcer

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Symposium

ANALYSIS OF CARCINOGENIC AIR POLLUTANTS

August 1962

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UNITED STATES OF AMERICA

Symposium

Analysis of Carcinogenic Air Pollutants

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INTRODUCTION

CHAIRMAN SAWICKI: We welcome our foreign visitors, the speakers, and all other participants to this meeting at Robert A. Taft Sanitary Engineering Center.

I would like to introduce the first speaker. He has been the driving force in initiating this meeting and a powerful factor in placing Government research in air pollution on a firm foundation. He is the Chief of the Laboratory of Engineering and Physical Sciences, which is sponsoring this meeting. Mr Arthur C. Stern.

MR. STERN: We are holding this symposium for a selfish reason. We want to tap the best brains in the world working on the problem to improve our own ability to measure carcinogenic air pollutants. We regret that our scientific colleagues from the USSR were unable to attend. We are proud to have assembled such a distinguished group of scientists and hope that the interchange of information will be beneficial to all.

In the United States the Federal department responsible for research in air pollution is the Department of Health, Education, and Welfare; its headquarters are in Washington, D.C. The Secretary of the Department of Health, Education, and Welfare is Abraham Ribicoff.

The organization within the Department of Health, Education, and Welfare responsible for this area of research is the Public Health Service, which is under the direction of the Surgeon General, Dr. Luther Terry, with headquarters also in Washington, D.C.

Within the Public Health Service the Bureau responsible for environmental health is our Bureau of State Services. It is under the supervision of Dr. Theodore Bauer and also has headquarters in Washington, D.C.

Last September a Division of Air Pollution under the direction of Mr. Vernon MacKenzie was set up within the Bureau. I have discussed our internal organization to orient those of you who came from abroad to our scheme of things. Later I will tell you where you are geographically.

Prior to 1953, air pollution problems were occasionally tackled as the part-time assignment of scientists normally otherwise engaged. For instance, the Public Health Service responded to the disaster in Donora, Pennsylvania, by curtailing some of the activities of our Division of Occupational Health and mustering a team therefrom to go into the field. This was done at the expense of other activities. As soon as the report on the disaster was completed, the team was disbanded and the members returned to areas of investigation other than air pollution.

In late 1953, the Sanitary Engineering Center received a contract from the Army Chemical Corps to determine the protein concentration of the atmosphere in a number of sections of the United States. The folks here who at that time set up the necessary sampling network recognized that the samples were useful for more than the determination of proteins. They therefore used portions of the sampling filters for the measurement of several other constituents of atmospheric particulate matter. This led in 1954 to the initial specific appropriation to the Public Health Service for air pollution work, *i.e.*, an intensification of this sampling effort, which was soon named the National Air Sampling Network.

In 1955, air pollution was recognized as a separate research entity at the Sanitary Engineering Center and an organization was created which grew from about 2 or 3 people in early 1955 to our present air pollution organization of over 200 people. The present budget for air pollution research, technical assistance, and training in the Federal establishment is $8,600,000, of which about 80 percent goes into research.

Our research efforts are divided about equally into three parts. About one third is research-grant activity, wherein funds are made available to individual researchers in universities to work on projects of their own choosing. These projects are carefully screened to determine whether they have adequate merit and adequate priority to receive funds. Research grants are not limited to citizens of the United States. We have provided research-grant funds to researchers in countries other than in the United States where over-all benefit to science will be the ultimate result. If any of you are interested in information on research grants, we will be glad to supply it.

Another third is contract activity, the largest part of which is with other Federal agencies. We have fairly substantial contractual arrangements for research with the U.S. Bureau of Standards, the U.S. Bureau of Mines, the U.S. Weather Bureau, the Department of Agriculture, the Tennessee Valley Authority, and the Library of Congress. In addition, there are substantial contracts with university-hospital groups and universities, mainly for toxicological and epidemiological types of studies.

The final third of our over-all activity is that which we do directly with our own staff and equipment. This activity is divided within the Division of Air Pollution into several operating branches.

A small branch located in Washington is responsible for research grants and training grants. Another is our Technical Assistance Branch. This has its headquarters in Cincinnati, and its mission is to translate the results of research and prior knowledge into the kind of information and assistance needed by air pollution control agencies in the States and by the many communities of the United States.

The remaining three branches are for research. One is our Field Studies Branch, primarily concerned with community studies involving both air quality measurement and epidemiological measurements to establish the relationship in that community between what we find out about the pollutants in the atmosphere and what we find out about the incidence of various diseases in the affected population.

The two remaining groups are primarily laboratory oriented. One is our Laboratory of Medical and Biological Sciences, which has its principal laboratory at the Sanitary Engineering Center annex building about 12 miles from the Sanitary Engineering Center in Cincinnati. This building has just recently been acquired and will become our principal research center for experimentation on the effects of air pollutants on laboratory animals. It will become a large establishment, with large animal colony facilities, and will, of course, have all the necessary supporting laboratories for chemical and physical analysis to provide biochemical and other types of support necessary for medical and biological studies. We have one small component of that group on our campus—the greenhouse and the Laboratory for Agricultural Research.

The Laboratory of Engineering and Physical Sciences, your host, is subdivided into several operating groups. One that I mentioned previously as being the precursor of all present air pollution activity in the Public Health Service is the National Air Sampling Network Section, which actually operates a multiplicity of National Air Sampling Networks. We are still continuing, at over 200 cities and a number of nonurban locations, the type of measurement that we initiated in 1954, based upon 24-hour samples through an 8- by 10-inch glass-fiber filter. These samples are taken on a biweekly basis and returned to this laboratory for a battery of tests and analyses. The total number of analyses we run on the samples that are most intensively analyzed is close to 30, whereas some of the samples get a much more cursory analysis. The intensity of analysis depends on our research interest at the particular time. We have more than 20,000 samples available as a library for any type of analysis we choose to make. We are continually in the process of assembling, collating, and publishing this type of data.

The second of the national networks is a gas sampling network in 50 cities, where again we are measuring on a biweekly 24-hour average basis. We measure sulfur dioxide and oxides of nitrogen and are piloting several other types of measurements.

We are in the process of setting up in 8 cities in the United States a continuous air monitoring network, which records every 5 minutes on punch paper tape a digitized concentration value for 7 gases in the air: carbon monoxide, sulfur dioxide, hydrocarbons, nitric oxide, nitrogen dioxide, ozone, and oxidants. The tapes are translated to punched cards and analyzed by computer.

The fourth of the networks for which the National Air Sampling Network is responsible is a precipitation network. In some 30 locations throughout the United States we take measurements of pollutants washed out of the air by rainfall.

The fifth of our national networks, one which is operated by our Meteorology Section is a turbidity-measuring network, based on viewing the sun at 0.5μ wavelength and determining the intensity of sunlight as it is diminished by particulate matter between the observer and the sun. This is a very sensitive measure with which we find that we can get information on concentration of particulates in the atmosphere as good as that from direct filter measurements.

Our meteorologists are assigned to us by the U.S. Weather Bureau and are concerned not only with the turbidity-measuring network, but also with the entire field of diffusion and dissemination of pollution. Their role is to explain how pollution gets from source to receptor and to give us mathematical models for that type of movement. We are more concerned with the multiple-source problem than with the single-source problem, although we are not without interest in what happens to the emission from a single stack.

The third of our operating sections is the Instrumentation Section, concerned with the development of all kinds of instruments needed by the various interests in air pollution, both laboratory and field.

The fourth section is our Engineering Research and Development Section concerned primarily with source problems. What is it that is emitted by a source of pollution; how do we measure it; how do we control it? Because of the prime interest in the United States at this time in automobile exhaust as a source of pollution, our main interest in Engineering Research and Development is research on the role of the automobile in the air pollution problem. We have here a pilot plant that generates polluted exhaust from automobiles, irradiates it in a chamber, and determines its effects on plants, animals, bacteria, visibility, and atmospheric chemistry.

Finally, there is the group that is your immediate host, the Chemical Research and Development Section, which is concerned with research into chemical methodology and the chemistry and photochemistry of air pollution, including, what brings you here, the chemistry of polynuclear hydrocarbons.

The Sanitary Engineering Center houses not only air pollution research activities but also works in the fields of water supply, water pollution, milk and food research, and radiological health. We also have a training activity concerned with all of these areas.

The question is frequently asked, "Why is all this in Cincinnati?" The reason is that the initial research in water supply and water pollution back in 1913 was on the Ohio River and was done from a houseboat. That houseboat eventually anchored at Cincinnati. The people left the boat, came on land, and established a research laboratory in downtown Cincinnati. In 1953 construction started on this building and the Center started here. We soon outgrew this building, started putting temporary buildings across the road and, as I previously mentioned, just this past year acquired another building about half this size 12 miles from here.

Before turning the meeting back to Dr. Sawicki, I would like to introduce two folks whom I think you should know. One is the Deputy Chief of the Laboratory of Engineering and Physical Sciences, Dr. John Ludwig; the other is Dr. A. P. Altshuller, the Chief of the Chemical Research and Development Section, the section with which Dr. Sawicki is associated.

CHAIRMAN SAWICKI: We hope that a little sandpaper will be applied constructively to the improvement of the present methodology. We hope that the speakers will set the tuning forks in many of our minds vibrating, and finally, we hope that the small Niagara of knowledge pouring out in this room and wherever else the participants meet at this symposium may help initiate further investigations in the air pollution field.

Mode of Formation of Carcinogens in Human Environment [1]

G. M. BADGER, *Organic Chemistry Department, University of Adelaide, Adelaide, South Australia*

SUMMARY

It is suggested that polycyclic aromatic hydrocarbons are formed at high temperatures from aliphatic hydrocarbons or from simpler aromatic hydrocarbons by free radical reactions. Carbon-hydrogen and carbon-carbon fission occurs to give "primary" radicals, and these recombine in various ways to give less hydrogenated, and more condensed, products. Acetylene, butadiene, vinylcyclohexene, styrene, ethylbenzene, butylbenzene, phenylbutadiene, tetralin, benzene, toluene, propylbenzene, and indene were all pyrolyzed at 700° C. The resulting tars were analyzed by distillation, gas-liquid chromatography, chromatography on alumina and on acetylated cellulose, and by infrared, ultraviolet, and fluorescence spectroscopy. In some cases about 50 different products were identified satisfactorily and their percentage yields determined. Consideration of the "primary" radicals and the yields of the various products suggests plausible mechanisms for their formation. Some of these mechanisms were confirmed by radiochemical analysis of products from the pyrolysis of (α-C^{14}) ethylbenzene. Tars from the pyrolysis of (α-C^{14}) tetralin and of (δ-C^{14})*n*-butylbenzene are under investigation.—NCI Monogr. No. 9: 1–16, 1962.

CANCER WAS first recognized as an industrial disease in 1775 when it was shown that chimney sweeps were especially liable to contract cancer of the scrotum. Later it was found that workers in the coal tar industry, in the shale oil industry, and in the cotton-spinning industry also had a higher incidence of skin cancer than the population at large. This led to the examination of soot, tar, and mineral oil for carcinogenic factors; in 1933, benzo[*a*]pyrene was isolated from coal tar and also was synthesized. At that time it seemed benzo[*a*]pyrene might be responsible for many of the industrial cancers, but no information was available to suggest that it might be implicated in "spontaneous" cancers in man. In recent years, however, benzo[*a*]pyrene and other carcinogenic hydrocarbons have been found widely distributed in human environment. Such compounds were identified in soot, carbon black, processed rubber, the exhaust gases of gasoline and diesel engines, coal gas, coffee soots, human hair wax, oysters and barnacles taken from polluted water, smoked food, snuff, tobacco, and cigarette and tobacco smoke (*1*). Relatively large concentrations of some carcinogenic hydrocarbons have also been found in the atmosphere of some cities. The work now reported was undertaken in an attempt to determine the mode of formation of benzo[*a*]pyrene and similar hydrocarbons.

---

[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

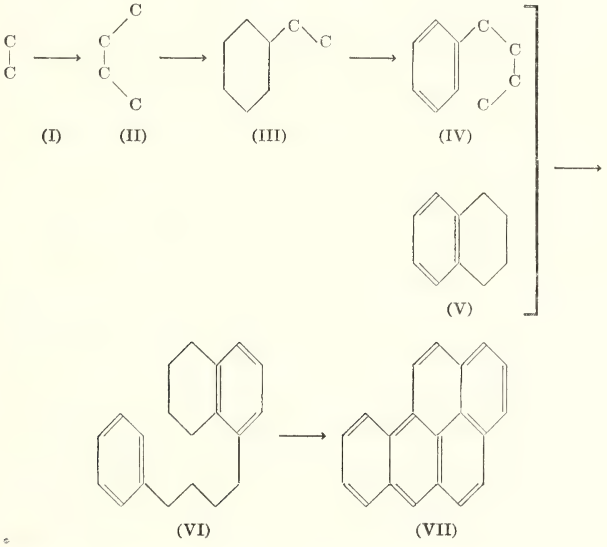
Though little information was available on which the initial work could be planned, it seemed that the carcinogenic hydrocarbons were formed by high temperature processes, sometimes involving incomplete combustion. For example, coal tar, produced by heating coal at 300 to 450° C in the absence of air, consists mainly of paraffins, cycloparaffins, olefins, and phenols and has only slight carcinogenic activity. Coal tar produced at higher temperatures, however, contains a greater proportion of polycyclic aromatic hydrocarbons and is much more carcinogenic (*2*). Similarly, shale oil obtained by solvent extraction of solid shale contained no benzo[*a*]pyrene, but the oil obtained by heating contained this hydrocarbon, and it is clear that it must be formed from some precursor during the heating process (*3*). Certain petroleums also become carcinogenic after being heated (*2*), and carcinogenic tars have been obtained by the pyrolysis of cholesterol, yeast, skin, acetylene, isoprene, dicetyl, and aliphatic hydrocarbons from tobacco (*1*).

Berthelot found that when acetylene is heated it forms a tar which contains benzene and other aromatic hydrocarbons; this has often been confirmed. Many years later, Kennaway (*2*) found that tar produced in this way has considerable cancer-producing power. The tar gave a fluorescence spectrum similar to that given by benzo[*a*]pyrene (*4*), and the presence of this hydrocarbon has been generally assumed. In a reinvestigation of acetylene tar, the presence of benzo[*a*]pyrene was confirmed, and indeed was present to the extent of 2 percent (*5*). This confirmation of the presence of benzo[*a*]pyrene may be important, for the incomplete combustion of organic compounds is known to give some acetylene, and acetylene has been detected in tobacco smoke (*6*). Many other polycyclic aromatic hydrocarbons were also identified.

The pyrolysis of acetylene was important in planning the present work for it clearly showed that chain lengthening can occur. A four-carbon unit such as vinylacetylene or butadiene seemed the most reasonable initial product; the pyrolysis of butadiene had also been shown to give ethyl benzene, styrene, naphthalene, tetralin, and many polycyclic compounds, presumably via the butadiene dimer, vinylcyclohexene (*7*).

Another critical observation is that tetralin, when treated with aluminum chloride, gives a complex mixture ("Schroeter tar") which becomes carcinogenic after being heated (*8–10*). This tar has a fluorescence spectrum similar to that of benzo[*a*]pyrene, and it has been suggested that this hydrocarbon is formed in the reaction by cyclodehydrogenation of 5-4′-phenylbutyltetralin. Schroeter tar certainly contains 6-4′-phenylbutyltetralin, and the formation of a small amount of the isomer seemed not unlikely. Here again reinvestigation has shown that benzo[*a*]pyrene is certainly in Schroeter tar, and its suggested mode of formation still seems entirely reasonable (*11*).

From the meager experimental evidence available, therefore, it was suggested as a working hypothesis for the present work that benzo[*a*]pyrene may be formed by a stepwise process as in (I)→(VII) (*1*). This hypothesis presupposed that benzo[*a*]pyrene would be formed by the pyrolysis of any of the intermediate compounds, but it did not imply that complex organic substances necessarily break down to a two-carbon fragment before resynthesis. Indeed, from the first, it seemed likely that C_6–C_4 fragments (IV and V) could also arise by cyclization of a C_{10} hydrocarbon such as *n*-decane or from a higher hydrocarbon, such as dicetyl, by cracking followed by cyclization.



(I) (II) (III) (IV) (V) (VI) (VII)

To test this hypothesis, several possible intermediate compounds (table 1, compounds marked with asterisk) and related compounds have been pyrolyzed at 700° C and the resulting tars analyzed by distillation, gas-

TABLE 1.—Compounds pyrolyzed at 700° C

Type	Compound
C_2	Acetylene*
C_4	Butadiene*
C_6	Benzene
C_6–C_1	Toluene
C_6–C_2	Vinylcyclohexene*
	Styrene*
	Ethylbenzene*
C_6–C_3	Indene
	Propylbenzene
C_6–C_4	Butylbenzene*
	Tetralin*
	Phenylbutadiene*
C_6–C_4, C_6–H_4	Phenylbutylnaphthalene*

*Possible intermediate compounds that have been pyrolyzed.

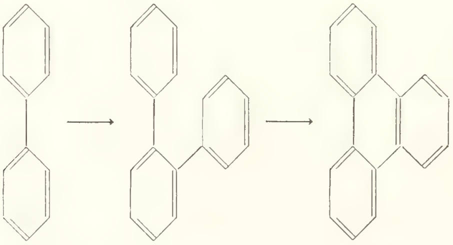
liquid chromatography, chromatography on alumina, acetylated cellulose, and acetylated paper (*12, 13*), and by ultraviolet, infrared, and fluorescence spectroscopy. As many as 50 different compounds have sometimes been identified, and their concentrations in the tar determined.

There can be little doubt that the high-temperature reactions leading to the formation of these tars are predominantly radical in nature. In any attempt to understand the mode of formation of polycyclic compounds from simpler hydrocarbons, it is therefore important to try to determine which bonds in the compound under pyrolysis are most likely to break to yield radicals. This is not impossible for bond dissociation energies, for various types of carbon-hydrogen and carbon-carbon bonds are known with some accuracy (table 2) (*14*). It may be noted that carbon-carbon bonds in aromatic rings and carbon-carbon double (and triple) bonds are much stronger than carbon-carbon single bonds.

TABLE 2.—Bond dissociation energies

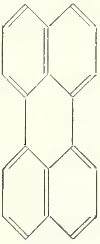
Bond	Bond dissociation energy (kcal/mole)
CH_3–H	101
C_2H_5–H	98
C_6H_5–H	102
$C_6H_5CH_2$–H	77. 5
CH_3–CH_3	83
C_2H_5–C_2H_5	78
C_6H_5–CH_3	89
$C_6H_5CH_2$–CH_3	63
$C_6H_5CH_2$–$CH_2C_6H_5$	47

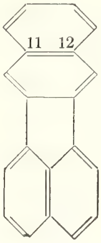
The first consideration is what happens to benzenoid rings on pyrolysis. When benzene itself was pyrolyzed at 700° C, much of the initial material was recovered unchanged and the major transformation product was biphenyl (VIII, 20.84%) (*15*). The only other substances formed in relatively large amounts were *p*-terphenyl (1.02%), *m*-terphenyl (0.37%), and triphenylene (X, 0.67%). It seems certain that fission of a C–H bond in benzene takes place, giving phenyl radicals, which then react with further benzene molecules to give biphenyl, and that further phenylation (and cyclodehydrogenation) gives the other compounds named. Some fission of the benzene ring did occur, as shown by the identification of methane and ethylene in the exit gases, and this accounts for the


(VIII) (IX) (X)

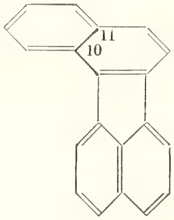
other polycyclic hydrocarbons which were present in very small amounts, totaling only 0.5 percent of the tar.

Somewhat similar results have been obtained, following the pyrolysis of naphthalene, by Lang and Buffleb (*16*). The major products were binaphthyls, presumably formed from naphthyl radicals and naphthalene, but further condensation can and did occur to give perylene (XI), 11,12-benzofluoranthene (XII), and 10,11-benzofluoranthene (XIII). Some or all of these compounds have also been identified in tars resulting from the pyrolysis of tetralin (*17*), alkylbenzenes (*18*), butadiene (*19*), and vinylcyclohexene (*20*), and it may be noted that naphthalene is also an important constituent of these tars.


(XI)

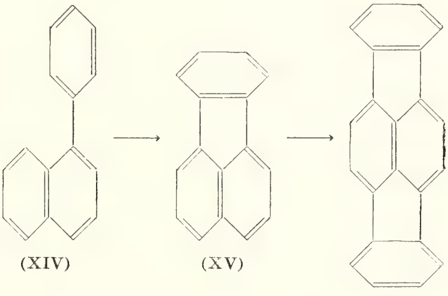


(XII)

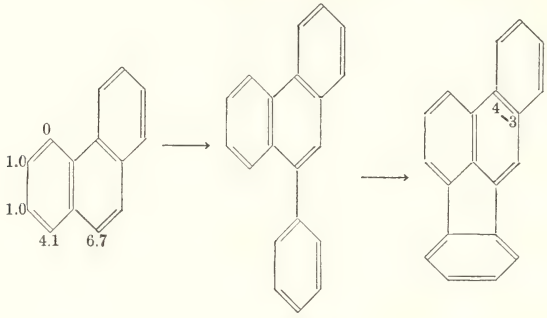


(XIII)

There is every reason to believe that substitutions with aryl radicals and cyclodehydrogenation reactions are involved in the formation of many other polycyclic aromatic hydrocarbons. The formation of fluoranthene (XV), for example, could occur by the phenylation of naphthalene to give 1-phenylnaphthalene (XIV) followed by cyclodehydrogenation; phenylation of fluoranthene to give 4-phenylfluoranthene followed by cyclodehydrogenation may account for the presence of 4,5-*o*-phenylenefluoranthene (XVI) in tars produced by the pyrolysis of propylbenzene, butylbenzene (*18*), and vinylcyclohexene (*20*).

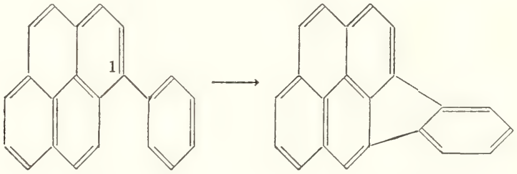

(XIV) (XV) (XVI)

Similarly, 3,4-benzofluoranthene (XIX) could be formed by the phenylation of phenanthrene (XVII) to give 9- (or 1-)phenylphenanthrene (XVIII) followed by cyclodehydrogenation. In this connection it is significant that the relative yields of phenylphenanthrenes obtained by phenylation of phenanthrene with diazoaminobenzene or benzoylperoxide have been determined and are given on the adjacent formula (XVII) (*21*). It seems that the reactivities of the positions in phenanthrene toward phenyl radicals are in the order $9>1>3\approx 2$.



(XVII) (XVIII) (XIX)

In the same way, the phenylation of pyrene would be expected to give mainly 1-phenylpyrene (XX) and on cyclodehydrogenation 1,10-(*o*-phenylene)pyrene (XXI) would be expected. This hydrocarbon has been identified in tars formed by the pyrolysis of alkylbenzenes (*18*), vinylcyclohexene (*20*), and butadiene (*19*). It has also been found after pyrolysis of a mixture of pyrene and benzene (*16*).



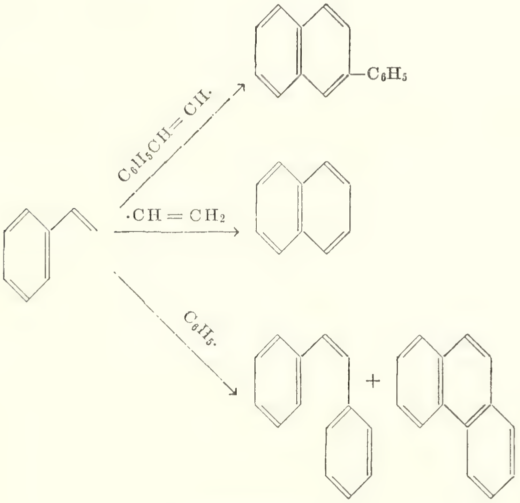
(XX) (XXI)

The dissociation energy of C–H bonds (to produce aryl radicals) is not inconsiderable, and a relatively high temperature seems required before this reaction proceeds to a significant extent. Few data are available at present, but it has been observed that the pyrolysis of acetylene at 700° C gave benzene (28%) and biphenyl (2.7%); pyrolysis at 530° C gave a similar yield of benzene and no biphenyl (*5*).

The pyrolysis of alkylbenzenes and alkenylbenzenes creates additional problems; in such compounds carbon-carbon bond fission can occur to a significant extent, as well as carbon-hydrogen fission. The expected reactions can be illustrated by reference to styrene (*22*).

It seems likely that the caraon-hydrogen bonds in styrene all have bond dissociation energies of the order of 100 kcal. If account is taken of their bond orders, approximate values for the bond dissociation energies for the carbon-carbon bonds can also be obtained. It seems that there is little difference between the energy required to break any C–H bond and the C–C single bond, but a much greater energy is required to break the carbon-carbon double bond or the aromatic ring. This leads to the conclusion that the most important primary radicals in the pyrolysis of styrene may be $C_6H_5\cdot$, $C_6H_5CH = CH\cdot$, $C_6H_5\dot{C} = CH_2$, $\cdot CH = CH_2$, and $\dot{C}_6H_4CH = CH_2$.

If styrene is attacked by a phenyl radical, then stilbene, bibenzyl, and phenanthrene might be expected; stilbene (1.3%), bibenzyl (0.9%), and phenanthrene (11.1%) were found in the tar. If styrene is attacked by a styryl radical, then 2-phenylnaphthalene would be expected; this compound was found (2.5%). If styrene is attacked by a vinyl radical, then naphthalene might be expected; this compound was also found (6.1%) in the tar (text-fig. 1). Abstraction of hydrogen by these radicals would account for the ethylene, benzene (6.4%), and ethylbenzene (1.6%) found; the presence of phenyl radicals would also account for the biphenyl (1.6%).

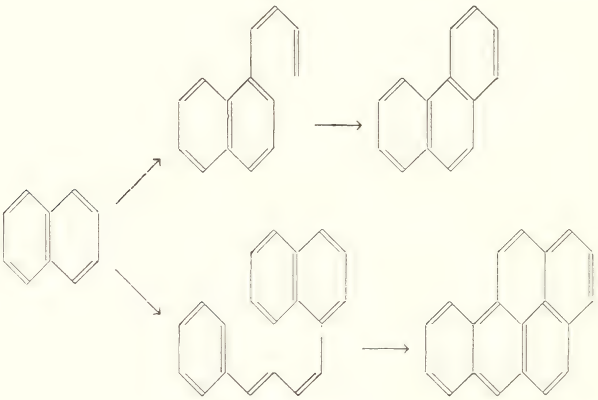


TEXT-FIGURE 1

The tar obtained from the pyrolysis of styrene also contained toluene (3.3%), and at first sight it might be thought that this was formed by fission of the carbon-carbon double bond followed by hydrogen abstraction. It is much more likely to arise from bibenzyl, the central bond of which has a very small bond dissociation energy (table 2). Fission would give two benzyl radicals, and toluene could be formed by hydrogen abstraction.

Similar arguments lead to the conclusion that phenylbutadiene will undergo bond fission at high temperatures to yield the following important "primary" radicals: $C_6H_5CH = CHCH = CH\cdot$, $C_6H_5\cdot$, $\cdot CH = CHCH = CH_2$, $C_6H_5CH = CH\cdot$, $\cdot CH = CH_2$, and $\cdot H$. The pyrolysis of phenylbutadiene yielded a tar containing a large amount of naphthalene (67.2%), and all the other products formed can be accounted for by secondary reactions involving these initial products (*23*).

Reaction of naphthalene with butadienyl radicals, or of styryl radicals with phenyl radicals would yield phenanthrene (found, 13.3%). Reaction of naphthalene with phenyl radicals would yield 1-phenylnaphthalene and (by cyclodehydrogenation) fluoranthene (found, 3.7%) and with phenylbutadienyl radicals (text-fig. 2) would yield phenylbutadienylnaphthalene and (by cyclodehydrogenation) benzo[*a*]pyrene (found, 0.12%). Similarly, further reaction of the phenanthrene formed would give additional products. Reaction of phenanthrene with phenyl radicals would give phenylphenanthrenes (found, 0.57%) and (by cyclodehydrogenation of 9- or 1-phenylphenanthrene) 3,4-benzofluoranthene (found, 0.78%). It is interesting that this tar contained no benzene, presumably because hydrogen abstraction by phenyl radicals was precluded, owing to the unsaturated nature of the compound being pyrolyzed, but biphenyl (5.2%) was found. It seems likely that the biphenyl was formed in this pyrolysis by a chain termination reaction between two phenyl radicals. It is also of interest that some triphenylene (0.83%) was also identified. This could arise by further phenylation and cyclodehydrogenation of biphenyl, or by the action of butadienyl radicals on phenanthrene.



Text-figure 2

Tars produced by the pyrolysis of indene (*24*), tetralin (*17*), vinylcyclohexene (*20*), and alkylbenzenes (*18*) provided further information. The C–H bonds in the methyl group of toluene have a bond dissociation energy of 78 kcal per mole; that for the $C-CH_3$ group is 89 kcal per mole. The initial decomposition of toluene at high temperatures would therefore be expected to occur predominantly by fission of a C–H bond to give a benzyl radical. For the other alkylbenzenes, fission of a C–C single bond is a lower energy process (60–80 kcal/mole) than C–H fission (90–100 kcal/mole) and the former would therefore be expected to predominate. Similarly, in vinylcyclohexene and tetralin the C–C single bonds must have bond dissociation energies around 80 kcal per mole, and these bonds would also be expected to undergo ready fission to give radicals.

The pyrolysis of alkylbenzenes would therefore be expected to give "primary" radicals such as $C_6H_5\cdot$, $C_6H_5CH_2\cdot$, $C_6H_5CH_2CH_2\cdot$, $CH_3\cdot$, $CH_3CH_2\cdot$, etc., and the products can be accounted for by secondary reactions involving these radicals. Benzene was found in every case, the largest amounts in tars formed by the pyrolysis of ethylbenzene and butylbenzene. Fission of the bond linking the side chain to the bulky aromatic nucleus might be expected to be increasingly preferred with increasing chain length, and there may also be a preference for the removal of a two-carbon fragment; this would explain the observation that propylbenzene gives a greater amount of toluene than either ethylbenzene or butylbenzene.

Chain resynthesis must proceed to an appreciable extent, for naphthalene is in the tars derived from all the alkylbenzenes studied and in those from indene and vinylcyclohexene. The probable mechanism of chain resynthesis may be illustrated by the following:

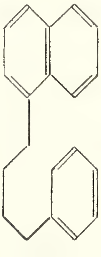
$$C_6H_5\cdot + CH_2{=}CH_2 \longrightarrow C_6H_5CH_2CH_2\cdot \qquad \ldots\ldots(1)$$

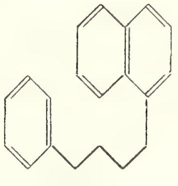
$$C_6H_5CH_2\cdot + CH_2{=}CH_2 \longrightarrow C_6H_5CH_2CH_2CH_2\cdot \qquad \ldots\ldots(2)$$

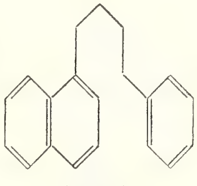
$$C_6H_5CH_2CH_2\cdot + CH_2{=}CH_2 \longrightarrow C_6H_5CH_2CH_2CH_2CH_2\cdot \qquad \ldots\ldots(3)$$

$$C_6H_5\cdot + CH_2{=}CH{-}CH{=}CH_2 \longrightarrow C_6H_5CH_2CH{=}CHCH_2\cdot \qquad \ldots\ldots(4)$$

Evidence for the feasibility of the final step (VI→VII) in the mechanism suggested as a working hypothesis has been provided by the pyrolysis of 1-4′-phenylbutylnaphthalene [XXII, XXIII, XXIV, the dehydrogenated analogue of (VI) (*25*)]. This compound can undergo cyclodehydrogenation in several ways, and small yields of 1,1′-binaphthyl, perylene, and benzo[*a*]pyrene were obtained on pyrolyses, together with toluene, naphthalene, and 1-methylnaphthalene, produced by bond fission. When heated with palladium charcoal at 360° C, it gave 1-phenylphenanthrene in good yield.

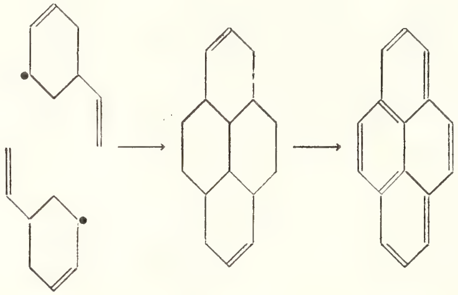

(XXII)


(XXIII)


(XXIV)

A detailed examination of the yields of various compounds in the different tars leads to the conclusion that the formation of benzo[*a*]pyrene from chrysene or from benz[*a*]anthracene by reaction with a two-carbon unit can be discounted (*26*). The pyrolysis of indene, for example, yields only a very small amount of benzo[*a*]pyrene, although the yield of chrysene was high and ethylene was present in the exit gases. On the other hand, many of the tars containing relatively large amounts of benzo[*a*]pyrene also contained large amounts of pyrene, *e.g.*, the tars from acetylene, butadiene, and butylbenzene and vice versa. It seems likely, therefore, that reaction of a four-carbon unit, such as butadiene, with a pyrene intermediate may also be an important route to benzo[*a*]pyrene, in addition to that represented by (I)→(VII).

A suitable pyrene intermediate could be formed by the dimerization of two vinylcyclohexene radicals (XXV). The resulting hydropyrene (XXVI) would give pyrene (XXVII) on dehydrogenation; addition of another molecule of butadiene followed by dehydrogenation would give benzo[*a*]pyrene.



(XXV) (XXVI) (XXVII)

Further evidence on the proposed mechanisms has been sought by pyrolyzing compounds labeled with radiocarbon. The pyrolysis of [αC^{14}]ethylbenzene has been completed; 10 products have been isolated in a pure state and in sufficient quantities for radiochemical analysis, and certain conclusions can be drawn from the results (table 3) (*27*).

Table 3.—The pyrolysis of [α-C^{14}]ethylbenzene

Product	Number of labeled atoms
Benzene	0. 024
Biphenyl	0. 045
Toluene	0. 74
Styrene	1. 07
Naphthalene	2. 00
Phenanthrene	1. 05
3,4-Benzofluoranthene	0. 91
Anthracene	1. 03
Pyrene	2. 4, 2. 65
Chrysene	3. 14

According to the previous discussion, ethylbenzene must be expected to undergo carbon-carbon bond fission in two main ways. The first (equation 5) would give a labeled benzyl radical and an unlabeled methyl radical. The second (equation 6) would give an unlabeled phenyl radical and a labeled ethyl radical which might also undergo further scission to give both labeled and unlabeled methyl radicals.

$$C_6H_5\overset{*}{C}H_2CH_3 \rightarrow C_6H_5\overset{*}{C}H_2\cdot + CH_3 \qquad \ldots\ldots(5)$$

$$C_6H_5\overset{*}{C}H_2CH_3 \rightarrow C_6H_5\cdot + \cdot\overset{*}{C}H_2CH_3 \qquad \ldots\ldots(6a)$$

$$\rightarrow C_6H_5\cdot + \cdot\overset{*}{C}H_3 + \cdot CH_3 \qquad \ldots\ldots(6b)$$

Any benzene or biphenyl formed in this pyrolysis, therefore, should be unlabeled. In fact the two products isolated were practically inactive, confirming the stability of the phenyl ring and also the view that breakdown and resynthesis of the benzene ring occurs only to a very small extent at 700° C. Indeed, the small amount of activity could have resulted from a small amount of benzene formed by the polymerization of three (labeled) two-carbon units (from equation 6a).

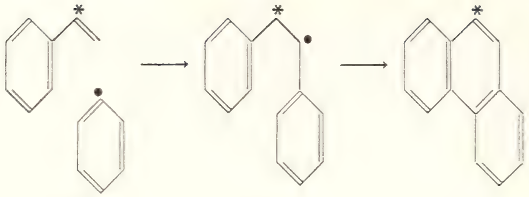
If the toluene formed in this pyrolysis is derived from the (labeled) benzyl radicals by hydrogen abstraction, then it should have activity corresponding to one labeled atom. On the other hand, if it is formed by reaction of a methyl radical with a phenyl radical (or benzene) and then if both labeled and unlabeled methyl radicals are present as suggested by equations 5 and 6b, it should have activity corresponding to less than 0.5 labeled atoms. Experimentally it was found that the activity corresponded to 0.74 labeled atoms, so both mechanisms must operate, the first predominating.

Fission of carbon-hydrogen bonds must also occur. Loss of one hydrogen would give a (labeled) phenylethyl radical, but loss of two hydrogens would give a (labeled) styrene molecule. As expected, the styrene isolated had activity corresponding to one labeled atom.

For the formation of naphthalene, chain resynthesis must be postulated. Reaction of a (labeled) phenylethyl radical with (labeled) ethylene would give a doubly labeled phenylbutyl radical (equation 7) and hence a doubly labeled naphthalene molecule. Several variants of this process are clearly possible, but it is significant that the naphthalene isolated had two labeled atoms.

$$C_6H_5\overset{*}{C}H_2CH_2\cdot + \overset{*}{C}H_2{=}CH_2 \rightarrow C_6H_5\overset{*}{C}H_2CH_2\overset{*}{C}H_2CH_2\cdot \qquad \ldots\ldots(7)$$

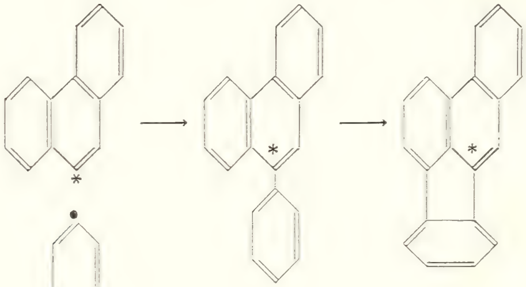
Two mechanisms for the formation of phenanthrene in this pyrolysis can be considered, and the present results make a clear-cut decision possible. If the phenanthrene is formed by a chain termination reaction between two (labeled) benzyl radicals or between a benzyl radical and toluene and subsequent cyclodehydrogenation of the resulting bibenzyl, then it should have two labeled atoms. On the other hand, if the phenanthrene (XXX) is formed by the action of a phenyl radical (unlabeled) on a (labeled) C_6–C_2 unit, such as ethylbenzene, styrene (XXVIII), a phenylethyl radical, or a styryl radical and cyclodehydrogenation of the product (XXIX), then it should have one labeled atom. Since only one labeled atom was found, the latter mechanism must be preferred. As a matter of fact, the former mechanism was earlier considered unlikely in view of the small yield of phenanthrene following pyrolysis of toluene. Moreover, in view of the small bond dissociation energy for the central bond in bibenzyl, carbon-carbon fission to give benzyl radicals again would be a much more likely process than carbon-hydrogen fission.



(XXVIII) (XXIX) (XXX)

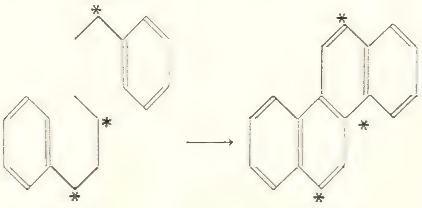
That the isolated anthracene also had only one labeled atom precludes the possibility of its formation from two (labeled) benzyl radicals. In this pyrolysis therefore it seems likely that the anthracene was formed by thermal rearrangement of the phenanthrene.

The 3,4-benzofluoranthene (XXXIII) isolated also had only one labeled atom. This result is in agreement with the mechanism postulated, namely, that this compound is largely formed by the phenylation (no label) of phenanthrene (in this case, one label) and cyclodehydrogenation.



(XXXI) (XXXII) (XXXIII)

In the pyrolysis of ethylbenzene the most likely route to chrysene would seem, on general grounds, to be by interaction of a C_6–C_2 unit with a C_6–C_4 unit (XXXIV). This would give chrysene (XXXV) having three labeled atoms; three labeled atoms were found. Other mechanisms are also possible, however, and since some of these would also give chrysene with three labeled atoms, no firm decision can be made, but the results are at least consistent with the proposed major route to this hydrocarbon.



(XXXIV) (XXXV)

It is also difficult to determine the route to pyrene, and the number of labeled atoms found (approx 2.5) suggests that more than one route may be important. A mechanism involving styrene as an intermediate would give pyrene having two labeled atoms; however, a mechanism involving the synthesis of pyrene from a C_2 unit via a C_4 unit and then vinylcyclohexene (XXV→XXVII) would give pyrene having eight labeled atoms. It must be tentatively concluded that both these mechanisms, or some variation of these, operate.

* * *

This work on the pyrolysis of hydrocarbons and analysis of the resulting tars has only been possible with the help of several skillful collaborators, R. G. Buttery, W. L. Kimber, G. E. Lewis, I. M. Napier, J. Novotny, and T. M. Spotswood, every one of whom has contributed to the success so far achieved. I should also like to express my thanks to the Damon Runyon Memorial Fund for Cancer Research, Inc., for financial support.

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DISCUSSION

DR. FALK: Dr. Badger, can you tell us something about benzo[*e*]pyrene and coronene formation from precursors? They are always in various types of soot, and I presume you must have found lots of them too.

DR. BADGER: I am sorry but I can not say much about this problem because there are quite a number of hydrocarbons for which we have not yet been able to devise mechanisms and benzo[*e*]pyrene is one of these. We are not sure indeed how pyrene itself is formed. Pyrene has 2½ labeled atoms and it seems, therefore, that it is formed by two different mechanisms, and we have not been able to isolate enough benzo[*e*]pyrene or benzo[*a*]pyrene in this experiment to be able to explain the mechanism. Coronene is only formed in very small amounts in all the pyrolyses that we have described. I do not know its mechanism of formation.

I should also mention that in recent months we pyrolyzed labeled tetralin and labeled butylbenzene (butylbenzene being a typical C_6–C_4 unit), with the label on the omega carbon atom. The benzo[*a*]pyrene was isolated in this case and had approximately two labeled carbon atoms so that this evidence supports our mechanism.

DR. VAN DUUREN: Dr. Badger, I would like to ask whether you have looked into the pyrolysis of aliphatic and alicyclic compounds, for example, steroids?

DR. BADGER: We have not done this yet. A few people have, of course, pyrolyzed some long-chain fatty compounds, such as the waxy material from tobacco. I think decane was done also some years ago but not under exactly the same conditions as our experiments. The information is incomplete yet, but it does look as if saturated compounds break down by carbon-carbon fission and that you can get a C_6–C_4 unit in some fashion to combine to form benzo[*a*]pyrene. We have not investigated the steroids, but hope to do so soon.

DR. THOMAS: Dr. Badger, I wonder if you could extrapolate from pyrolysis to self-sustaining combustion of internal combustion with respect to the proposed mechanism.

DR. BADGER: This is a little difficult to answer, but my reaction would be that the same mechanism would operate if the necessary free radicals were available, and under these conditions you would get similar reactions occurring.

DR. KATZ: I am sure, Dr. Badger, that you have considered the possibility or the formation of polycyclics through polymerization reactions involving the free radical hypothesis. These reactions could possibly take place at lower temperatures than the actual pyrolysis. Do you think there is anything in this hypothesis?

DR. BADGER: The only information we have indicates that polycyclic aromatic hydrocarbons are not obtained at low temperatures to any significant extent.

DR. KATZ: The free radical theory is one of the important theories in polymerization. I was suggesting that initially free radicals are formed, which then form the polycyclic compounds through polymerization.

DR. BADGER: Do you, in fact, get free phenyl radicals? I just wondered if the only radical you get is by opening a double bond. Do you ever get a radical with the loss of a hydrogen atom?

DR. KATZ: You do get that in polymerization reaction because one of the ways of explaining polymerization is through the formation of a free radical which involves loss of a hydrogen atom.

DR. LINDSEY: I should like to ask two questions. First, has Dr. Badger produced any quantities of carbon during his pyrolysis, and second, has he used temperatures other than the 700° C which he has mentioned?

DR. BADGER: The amount of carbon varies considerably depending on the hydrocarbon that is pyrolyzed. The amount of carbon formed in the pyrolysis of acetylene is very great indeed. In fact, we had a lot of trouble with acetylene in that the tube tended to block up. The experiment had to be stopped until the tube could be cleaned out. With some other hydrocarbons the quantity of carbon is relatively small. So the answer is that we did find carbon in varying amounts in all our experiments.

For the second question in connection with temperature, we have used 700° for all the pyrolysis experiments except for one or two experiments in which we investigated pyrolysis at 550°. The main difference was that the reactions involving carbon-hydrogen fission occurred to a much smaller extent at the lower temperature. Carbon-hydrogen fission requires a high temperature.

DR. SAHNI: I would like to know if there has been any theoretical work on the different mechanisms that have been suggested by Dr. Badger.

DR. BADGER: No. We try in a theoretical fashion to predict which bonds will break, and we have carried out determinations or calculations of bond orders to facilitate us in this effort. That is, if a carbon-carbon bond has a high double-bond character we think it is less likely to break than one with a low double-bond character. But apart from this, no.

DR. LYONS: Recently scientists at Woods Hole reported the discovery of considerable quantities of aromatic hydrocarbons in Massachusetts soil samples supposedly uncontaminated by pollution. In one sample approximately 3 percent benzo[*a*]pyrene was found. They believed that these aromatic hydrocarbons had a geophysical genesis. Could Dr. Badger suggest any possible mechanism in this regard?

DR. BADGER: No, but I am very interested in this. There is one particularly well-known example of a polycyclic aromatic hydrocarbon occurring naturally with a mercury ore. When you distill the mercury you obtain this hydrocarbon. Its formation is a very interesting scientific problem.

Some of the polycyclic aromatic hydrocarbons in soil could, of course, be produced by degradations from steroids, but this is complete speculation.

DR. THOMAS: You made a statement concerning chain synthesis, and it seems that side chains can be built up without too much difficulty. We have not been working in the pyrolysis region, but we have been working in the self-sustaining combustion region. In attempting to find alkyl derivatives of polycyclic hydrocarbons we have been unsuccessful. Could you comment on this particular point?

DR. BADGER: Yes, I think that in pyrolysis reactions wherein air pollutants are formed we very seldom get polycyclic compounds having alkyl substituents. We frequently find toluene, and a little methylnaphthalene, but hardly ever any significant quantities of the methylated pyrenes. We think this is because the alkyl substituent tends to come off under the pyrolytic conditions; that the bond between the aromatic ring and the saturated carbon is the weakest bond in the compound and consequently the alkyl group is lost as an alkyl radical.

Nevertheless, I think such compounds must exist even if only for a brief time. It might depend on the extent or the period the hydrocarbon was at the high temperature. There is no doubt that the chain resynthesis does occur, because we do get naphthalene from substances like toluene but we do not find much methylnaphthalene.

DR. THOMAS: We have worked on the assumption that the bond energies were too high to form these side chains, where you have worked from the assumption that a relatively lower energy is needed to break the bond.

DR. BADGER: If you pyrolyze a methylated polycyclic compound, I think you will find that the methyl group will come off.

CHAIRMAN SAWICKI: Has anybody here isolated methylated hydrocarbons from any pollution source? Dr. Van Duuren, would you have a comment?

DR. VAN DUUREN: We have isolated several methylpyrenes from cigarette tars, and I believe that this work has been done before us by Dr. Lindsey and subsequently by Dr. Hoffmann. As I recall there were two or three of the methylpyrenes present in cigarette tars in small quantity.

DR. HOFFMANN: In answer to Dr. Lyons' question you mentioned the possibility that in the soil polynuclear aromatic hydrocarbons could be formed from steroids. I would like to have you amplify your remarks, please.

DR. BADGER: The hypothesis of polycyclic aromatic hydrocarbon formation from steroids was advanced in 1935, I think. To my knowledge there has been no evidence presented since that time that this transformation does in fact occur. Nevertheless, I think it is possible that you might get polycyclic aromatic hydrocarbons and steroids in the soil by degradation, sunlight, and other factors.

Apparatus for Determining the Contribution of the Automobile to the Benzene-Soluble Organic Matter in Air [1]

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SUMMARY

Part I describes condensation-filtration systems for collecting particulate matter from 100 percent of the exhaust gas and blowby gas of a modern automobile gasoline engine operating in a dynamometer installation. Exhaust gas flow rates for a simulated city-driving schedule of operation are given. Extraction apparatus for the recovery of the benzene-soluble fraction is described. Part II covers the design and testing of a mobile unit for the collection of particulate matter from the atmosphere. A centrifugal fan driven by an electric motor draws air through a 30 × 24 × 11½ in. "absolute" filter at the rate of 5000 cfm. A Pitot tube is utilized to measure air flow. Carbon monoxide concentration is measured continuously with a nondispersive infrared analyzer. The equipment is installed in a specially adapted Step-Van truck.—NCI Monogr. No. 9: 17–57, 1962.

THE INCIDENCE of lung cancer has increased greatly since 1900. Some estimates place the present mortality rate at 30 times that of 60 years ago (*1*, *2*). Although careful epidemiological studies point to a cause and effect relationship between the probability of contracting this disease and the degree of cigarette smoking, other factors must be sought to explain the higher mortality also observed among nonsmokers (*3–8*). The most commonly suggested factor is that of general air pollution, particularly in urban areas.

It is now well established that the automobile may contribute significantly to the concentration of hydrocarbons of low molecular weight and other organic materials in the air over cities (*9*, *10*). Not so well established is the contribution by the automobile to the concentration of organic materials of high molecular weight, particularly those of known or potential carcinogenic activity.

General Motors has embarked upon a cooperative program with the Sloan-Kettering Institute to assess the contribution of potential carcinogens by automobiles to the atmosphere (*11*, *12*). In this joint venture, the recovery of organic materials of high molecular weight, hereafter called "tar," as particulate matter from engine effluents and from the atmosphere is being carried out by General Motors personnel. Chemical analyses of the tar thus recovered and the biological assays for its tumor potency are carried out at the Sloan-Kettering Institute.

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[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

This paper comprises descriptions of the equipment and procedures used at the General Motors Research Laboratories for the collection of particulate matter from 100 percent of the exhaust gas and blowby gas of a modern 364 in.^{3} engine and from the atmosphere. The paper is divided into two parts. The first part covers the development of apparatus and procedure for the recovery of tar from the exhaust gas and blowby gas of an automobile gasoline engine operating on a simulated city-driving schedule in a dynamometer installation. The second part describes the design and testing of a mobile sampling apparatus for the collection of particulate matter from the atmosphere and the recovery of tar from this material.

In this paper, continual reference is made to "tar," which is defined as the benzene-soluble organic material that is not volatile at 35° C and 0.1 mm Hg absolute pressure.

CONTRIBUTION OF THE AUTOMOBILE

Two general methods can be used for arriving at an assessment of the contribution of automotive exhaust gas to atmospheric carcinogens. These are:

Direct technique.—In this method the rate of emission of carcinogenic material per automobile hour or per gallon of gasoline consumed is determined experimentally. By multiplication of this intensive quantity by the proper extensive quantity (either the number of hours of automobile operation or the total quantity of gasoline consumed), the total amount of carcinogens in the atmosphere, due to automobiles, can be estimated.

Modification of this technique permits assessment of the expected concentration of carcinogenic matter in the atmosphere attributable to the automobile. For example, one can relate the automotive emission rates of certain carcinogens and carbon monoxide. By measurement of the atmospheric carbon monoxide concentrations, the expected atmospheric concentrations of these carcinogens can be estimated on the basis that the automobile is the exclusive source of carbon monoxide. A comparison of these carcinogen concentrations with the measured atmospheric concentrations provides an estimate of the relative contribution of the automobile and other sources. A similar analysis can be made with lead rather than with carbon monoxide, based on the assumption that automobile emissions are the exclusive source of lead.

Another direct method for determining the relative contribution of automobiles is the comparison of analyses for certain polynuclear aromatic hydrocarbons in exhaust gas tar and atmospheric tar. In this connection it would also be desirable to analyze effluents from other possible sources of carcinogens to pinpoint their contribution to the atmosphere.

Indirect technique.—In this method the concentration of carcinogenic material in the atmosphere is measured at a number of locations representative of different automobile traffic densities. The variation in the concentration of carcinogenic hydrocarbons with traffic density permits an indirect estimate of the contribution of the automobile. With this technique, measurements of atmospheric concentrations of carbon monoxide and lead can be used as the criterion of traffic density. Since these components are definitely related to exhaust gas emission, they may serve as a better index of vehicle emissions than do simple traffic counts.

To pursue the program by both direct and indirect methods, equipment was constructed for the collection of tar from exhaust gas and blowby gas of an engine, and a mobile apparatus was built for the collection of particulate matter from the atmosphere.

PART I. APPARATUS FOR THE RECOVERY OF BENZENE-SOLUBLE TAR FROM EXHAUST GAS AND BLOWBY GAS OF AN AUTOMOBILE ENGINE

Quantitative relationships are desired.—One important objective of the engine experimentation is the determination of the relationships between the quantity of tar collected and the volume of exhaust gas or blowby gas, the quantity of fuel used, and the time that the engine is operated. As far as can be determined, no one has carried out the recovery of tar from exhaust gas of an engine in a manner that would yield these relationships precisely. Kotin and co-workers (*13, 14*) have reported the time rate of emission of certain polynuclear aromatic hydrocarbons from both gasoline and diesel engines. However, no description of collection methods is given, and it appears that only filtration of exhaust gas through filter paper was utilized to capture the relatively nonvolatile hydrocarbons and other materials.

Mittler and Nicholson (*15*) recovered a condensed water phase from the exhaust gases of both a gasoline and a diesel engine. The condensed water phase was extracted with benzene. The exhaust gases were not filtered. The absolute volumes of exhaust gas sampled were given, but these were not related to the engine exhaust gas rates. Tar recovery rates were not given.

Scholl and co-workers (*16*) condensed both aqueous and oily liquid phases from exhaust gas of an engine operating on gasoline containing 5 percent lubricating oil. Exhaust gas flow rates were not given.

Bailey, Javes, and Lock (*17*) utilized a series of three traps at temperatures from 0° to —65° C to capture material from the exhaust gases of two diesel engines in road operation. No filter was used. Sampling rates were measured, but the volumes of exhaust gas sampled were unrelated to the emission rates from the engines. However, these authors reported grams of "oil" per 1000 ft^3 of exhaust gas, as well as the concentrations of certain polynuclear aromatic hydrocarbons in the exhaust gas. In a bench-test engine installation, these same authors sampled 8 percent of the exhaust gas through a filter bag that was presumably later extracted. No condensation was carried out.

Marterstock and Reuter (*18*) collected soot from known volumes of exhaust gas sampled from a diesel engine operated at various loads. They used an asbestos paper filter to capture the soot from the exhaust gas, but they did not condense a liquid phase.

Stenburg, von Lehmden, and Hangebrauck (*19*) described techniques for the quantitative collection of benzo[*a*]pyrene in combustion gases, including automobile exhaust gas. Condensation followed by filtration through an absolute filter was used. No attempt was made to correlate the quantity of benzo[*a*]pyrene recovered with engine variables.

These earlier data are valuable in that, taken together, they conclusively demonstrate the presence of carcinogens in the exhaust gases of both gasoline and diesel engines. However, they provide little information as to the quantitative aspect of the problem. The principal aim of the work being carried out jointly by General Motors and the Sloan-Kettering Institute is to establish the quantitative output of carcinogenic materials from automobiles and to determine the total contribution of such materials to the atmosphere.

Apparatus and Procedure

Program begins as small-scale operation.—Initial exhaust gas tar collection operations were carried out on a relatively small scale. The objectives of the small-scale operations were twofold:

> The development of the most suitable method and materials for the quantitative recovery of particulate matter in exhaust gas. These tests included evaluation of both filter media and condenser requirements for use with exhaust gas
>
> The approximate measurement of the benzene-soluble tar in exhaust gas so that the volume of exhaust gas which had to be handled could be suitably compromised between equipment size and running time

In these early tests, the engine was operated at the equivalent of 35 miles per hour (mph) road load. Approximately 7 percent of the exhaust gas was sampled, with the use of a Penberthy ejector to withdraw the sample gas from the exhaust pipe at a point close to the engine. Arrangement of the apparatus was varied. In some experiments exhaust gas passed first through a filter and then through a condensing section, while in others the filter was placed after the condensing section. In general, the results of these experiments indicated that:

> Organic materials of high molecular weight in exhaust gas could best be recovered first by condensation of a water phase (some tar is also condensed as a separate layer), followed by filtration of the relatively dry gas
>
> A ½ in.-thick all-glass filter medium of very fine fibers could be used to retain the particulate matter in exhaust. This filter medium was selected because the initial pressure drop is only about 25 percent of the pressure drop of the somewhat superior "absolute" filter papers. It was determined that the ½ in.-thick me-

dium retained about 96 percent by weight of the exhaust gas particles captured on "absolute" paper

Approximately 2 g of tar per 1000 ft^3 of exhaust gas could be recovered at 35 mph road load

Approximately ⅓ of the total tar was recovered by extraction of the condensed water. It is important, therefore, that this fraction be collected

To supply tar at the rate required for the biological program, equipment to handle 100 percent of the exhaust gas should be built

Full-scale exhaust-treating system is built.—Based on information gained in the small-scale sampling operation, a combined condensation-filtration apparatus was constructed to handle 100 percent of the exhaust gas of a 250 horsepower (hp), 364 in.^{3} V-8 engine. This is the only engine utilized in the study to date. The system is illustrated in figure 1.

The T-shaped diverter valve (V_1) ahead of Precooler #1 is 4 by 4 in. in cross section and is powered by an air cylinder. It functions to direct exhaust gas either through the particulate collection system or to waste. When it is desired to collect tar during a specific event of the driving cycle, the valve is opened to the collection system; the remainder of the time the gas is diverted to waste.

Precoolers #1 and #2 are pipes 2½ in. in diameter by 5½ ft long, equipped with water jackets. With the engine operating on a relatively light-duty driving schedule, water is circulated only through Precooler #2. During the driving cycle, exhaust gas enters Precooler #1 at temperatures from 515 to 1000° F and leaves Precooler #2 at temperatures of 175 to 355° F.

The condenser is of shell and tube design, 6 in. in diameter by 6 ft long, and contains 286 one-quarter inch diameter tubes. Exhaust gas is passed on the tube side and cooling water on the shell side of the condenser. The high efficiency of the condenser is indicated by the fact that at all exhaust gas rates and temperatures encountered in the driving cycle the temperature of the gas leaving the condenser nearly equals the temperature of the incoming cooling water. The water temperature ranges from 58 to 71° F. The pressure drop across the clean condenser at the maximum gas flow occurring in the driving cycle is approximately 60 mm of water and increases to about 250 mm during approximately 80 hours of operation on leaded gasoline.

Water, tar, other liquids, and solid matter are removed from the exhaust gas in the condenser and are collected in the accumulator, which has a capacity of approximately 20 gallons. The condensed water phase is pumped periodically from the accumulator to a 55 gallon stainless steel drum for transfer to another location where the extraction operation is carried out.

After passing through the condenser, the exhaust gas passes over a baffle to the dry filter. At this point in the system, the exhaust gas is above its dew point. The filter unit has 5 pockets for the ½ in.-thick downlike filter medium. Before the filter material is installed, it is heated in a furnace at 700° F for 2 hours to destroy the phenolic binder and any other substances that would be soluble in the extraction solvent and thus would contaminate the tar recovered from the filter. In most experiments, 4 of the filter pockets are utilized and provide 26 ft^2 of filter area. Face velocity at the filter is less than 6 ft per minute at the maximum exhaust rate, and the pressure drop across the clean filter is about 2 mm of water at maximum exhaust flow. After 80 hours of operation on leaded gasoline the pressure drop increases to about 4 mm of water.

Photographs of the engine and complete tar collection system, the exhaust line T-valve, and the exhaust filter unit are shown in figures 2 through 4.

Tar is also collected from blowby gas.—The volume of blowby gas (the gas that passes the piston rings from the combustion chamber to the crankcase) ranges from about 1½ percent of the exhaust gas volume under cruise and acceleration conditions to as high as 10 percent under part throttle deceleration. Bennett *et al.* (*9*) have shown that the composition of blowby gas is 75 to 80 percent carbureted mixture and 20 to 25 percent combustion products. Since the composition of blowby gas is different from that of exhaust gas, it was decided to recover separately the nonvolatile fraction of blowby gas for analysis for polynuclear aromatic hydrocarbons.

Apparatus for the recovery of tar from blowby is shown schematically in figure 5. The equipment for the recovery of tar from blowby gas is basically the same as for exhaust gas, except that an aspirator is utilized to draw the blowby gas through the collection system. This maintains the pressure at the draft tube of the engine close to atmospheric pressure. Solenoid valve S–1 is only opened to handle the greater blowby rates occurring during accelerations. At idle and during decelerations, when the absolute blowby rate is minimum, the aspiration rate is slightly in excess of the blowby rate so that some fresh air is drawn through the engine. This condition may result in some extra oil mist being carried out of the engine, although the situation here is somewhat comparable to the action of the draft tube of a car traveling more than 30 mph.

Engine-dynamometer installation.—The engine used in these studies is connected to an electric dynamometer through a torque converter transmission. The dynamometer is equipped with electronic controls which permit control of "car" speed and load and simulation of vehicle inertia.

Driving cycle.—So that tar collected for analysis and use in biological experimentation may be as nearly representative of that produced in normal city driving, the engine is operated on a simulated city-driving schedule. The driving schedule, or cycle, is arbitrary, but intentionally approximates the relative amounts of time at idle, acceleration, cruise, and deceleration used in the California cycle (*20, 21*). This driving schedule, utilized in the dynamometer operation, is shown in table 1 (*see also* fig. 6). The exhaust gas rates and volumes are considered to be accurate to ±5 percent.

Accelerations are accomplished at constant throttle position. Intake manifold vacuums range from 10 to 12 in. of mercury during the 0 to 30 mph acceleration to 5½ to 7 in. of mercury during the 30 to 50 mph acceleration. Decelerations are at closed throttle with light braking.

TABLE 1.—City-driving schedule for tar collection program

Event	Driving condition (mph)	Duration		Exhaust rate (cfm)	Exhaust volume (ft^3)
		Seconds	Percent		
1	Idle	30	20	13. 6	6. 8
2	Acceleration, 0–30	15	10	76. 5	19. 1
3	Cruise, 30	30	20	40. 4	20. 2
4	Acceleration, 30–50	10	6. 7	145. 7	24. 3
5	Cruise, 50	15	10	62. 0	15. 5
6	Deceleration, 50–20	20	13. 3	13. 2	4. 4
7	Cruise, 20	20	13. 3	22. 8	7. 6
8	Deceleration, 20–0	10	6. 7	13. 2	2. 2
Total per cycle		150			~100
Average 23 mph				~40	

The engine is driven through the city-driving schedule by means of a "black box," called a Program-O-Trol (fig. 7), devised for this operation. Control features incorporated into the Program-O-Trol permit selection of event time, dynamometer torque, and throttle position. Each parameter can be regulated independently of the others for each of 10 events. The total cycle time is 150 seconds, and the cycle is automatically repeated.

Fuel and oil consumption rates are checked periodically. With gasoline and standard carburetion, the fuel consumption rate on the city-driving schedule is 13.2 lb per hour, which is equivalent to 11 miles per gallon. The oil consumption rate is about 1,600 miles per quart. Both rates are considered typical of a heavy automobile operated on the road according to this schedule.

Exhaust gas flow rates are measured directly and are also calculated from fuel rate measurements and air-fuel ratio determinations from Orsat analyses.

Tar is recovered from exhaust system with solvents.—At the conclusion of a run, tar in the exhaust system is recovered in 3 fractions:

From the condensed water phase by extraction with benzene
From the filter medium by extraction with a mixture of benzene and methanol
From the condenser, accumulator, transfer drum and other parts of the exhaust system by rinsing with toluene and, also, from solids recovered from the condenser and accumulator, by extraction with benzene

Tar in the condensed water phase (which has a *p*H of about 3) is extracted with benzene in a York-Scheibel 2-in. diameter, 11-stage countercurrent liquid-liquid extraction column. This apparatus is shown in figure 8. The water-phase feed rate is 13.9 liters per hour and the benzene feed rate is 1.4 liters per hour. The extraction of one drum (55 gallons = 208 liters) of condensed water phase is accomplished in 15 hours. Extraction of the tar from the water is essentially complete by this method, inasmuch as no additional tar is recovered by further contact with fresh benzene.

The filters are extracted in a large steam-heated, 17 liter, stainless steel Soxhlet apparatus (fig. 9). All 4 filter blankets are extracted at the same time. The extraction solvent is a mixture of benzene and methanol (4:1), and extraction time is 28 to 30 hours. About 20 syphoning actions occur during the extraction process.

All solvents are distilled through packed distillation columns at 2:1 reflux ratio before use.

Solvent removal is completed under vacuum.—Most of the solvent from the several tar solutions is stripped from the tar by distillation through Oldershaw or Podbielniak distillation columns operated at 2:1 reflux ratio. When the tar solutions are reduced to 500 to 700 ml, they are filtered through either Reeve-Angel X-984 glass-fiber filter paper or through a Millipore type HA membrane (0.45 μ pore size) with a Millipore filtration apparatus. The remaining solvent is removed under vacuum at room temperature, with the use of a flask-oscillating device (fig. 10) to hasten the evaporation of solvent. The final evaporation condition is 30 to 35° C at 0.05 to 0.1 mm Hg absolute pressure for a minimum of 3 hours.

Tar is recovered from the condenser and from the filters of the blowby system in the same manner as from comparable components of the exhaust gas system, except that wire brushing at the condenser is not required.

Results

It is not intended at this time to discuss the results of the experiments conducted with the equipment described. Details as to tar recovery rates from both exhaust gas and blowby, as well as a preliminary assessment of the contribution of the automobile to atmospheric carcinogens, were presented at the Society of Automotive Engineers Meeting in Detroit, January, 1962. Progress on the chemical and biological studies with tar recovered during operation with leaded gasoline is described by Hoffmann and Wynder (*22, 23*).

Operations with the equipment have provided detailed information on tar emission rates and a convenient means for the generation and collection of large quantities of exhaust tar. Furthermore, a number of important variables affecting both the quantity and composition of the tar generated have become apparent.

PART II. DESIGN AND TEST OF A 5000 CFM MOBILE AIR SAMPLING APPARATUS

Design of Sampling Equipment

Basic requirement is 1 g of tar in 10 hours of sampling.—The basic design requirements for the General Motors mobile air sampling laboratory was the capability of collecting 1 g of benzene-soluble material (tar) from the atmosphere in a period of approximately 10 hours. The sampling rate required for collection of 1 g in 10 hours depends on the concentration of tar in the atmosphere. Concentrations of benzene-soluble material in urban atmospheres, as determined by the National Air Sampling Network (*24*), were used for the estimation of the required sampling rate. These concentrations ranged from 0.5 μg per m^3 to 95.2 μg per m^3, with a national urban average of 11.3 μg per m^3. If one assumes collection and extraction efficiencies equivalent to those of the National Air Sampling Network, it is necessary on the average to sample 3,120,000 ft^3 of air to recover 1 g of tar. Therefore, the collection of 1 g of tar in 10 hours requires a sampling rate of approximately 5000 cfm. This rate is roughly 100 times greater than that of the so-called "high volume" samplers used by the National Air Sampling Network (*24*) and is 5 times greater than the rate reported for a sampler used by the General Motors Industrial Hygiene Department (*25*).

Operational requirements of the sampling apparatus.—In addition to the rate requirement, the following operation requirements dictated the design of the equipment:

The components making up the collection system must not contribute any tar to the material collected from the air
The apparatus must be capable of operating on 3-phase electrical power
The entire sampling unit should be mobile
The sampling equipment should be readily transferable from the vehicle in which it will usually operate to a building rooftop or other location
The equipment must be quiet

Basic sampling elements are filter, fan, and flow-measuring device.—Three basic components required for sampling particulate matter from the atmosphere are:

An element for removing and retaining the particulates from the air
A means of moving the air through the collection element
A means for determining the air volume sampled

The basic components chosen for the 5000 cfm sampling system are:

An all-glass, "absolute" filter
A centrifugal fan
A Pitot tube and differential manometer

Of the 3 basic components, the greatest effort was expended in the selection of the filter, the flow characteristics of which were needed in order to specify a fan and to design the system ducting.

"Absolute" filter selected for collecting particulate matter.—The general requirements of a filter are:

High efficiency
Minimum amount of impurities
Low resistance to flow
Sufficient strength to withstand pressure drop due to flow
Slow pressure buildup with particle deposition

The choice of an all-glass "absolute" filter medium for the particulate-collecting element was based on two factors: 1) successful experience with such "absolute" filter papers as reported by the National Air Sampling Network and the General Motors Industrial Hygiene Department and 2) consideration of the size distribution for particulate matter dispersed in the atmosphere (*26*).

Figure 11 illustrates the size ranges of a variety of particles found in the atmosphere. Particles as small as 0.001 μ are present, but the weight percentage of particles less than 0.01 μ is negligible. It is interesting that lung-damaging particles range from 0.25 to 10 μ (*7*, *27*) and that the optimum particle size for retention in the lung is between 1 and 3 μ (*28*).

To retain these small particles from the atmosphere, the filter medium should have a high efficiency for particles as small as 0.01 μ. Filters having this capability are classified as "absolute" filters and are available in two general types: 100 percent all-glass fibers or a mixture of glass and asbestos fibers.

Typical filter unit construction and specifications are shown in figure 12. The filter medium is sealed within the steel frame by a silicate sealing cement. To obtain a large filtration surface for a small filter frontal area (*e.g.*, a 24 × 30 × 11½ in. filter unit with only 5 ft^2 of frontal area contains about 280 ft^2 of filter paper), the medium is folded back and forth along the flow axis to form pleats. These pleats are supported by corrugated aluminum separators, which also channel the air through the unit.

The collection system was designed to utilize a single 24 × 30 × 11½ in. filter. Because the pressure drop through the single filter at a flow rate of 5000 cfm was beyond the range of data available from the manufacturers, this information was obtained by direct measurement. In addition, an investigation of the possible contaminants from the various filter papers was carried out.

A Flanders filter was selected as the sampling element because it had the lowest pressure drop and the least benzene-soluble impurities. The resistance of a Flanders unit at a flow rate of 5000 cfm was 4.0 in. of water. The benzene-soluble material in an unused filter, determined by extraction of 280 ft^2 of filter paper, was equivalent to 1.43 mg per ft^2. It has been determined that the soluble material in the filter paper does not interfere with analyses for polynuclear aromatic hydrocarbons.

Fan selection based primarily on lowest noise generation.—It was anticipated that permission to carry out sampling operations in residential areas would depend on the sound generated. Therefore, it was desirable that the equipment operate quietly. Since the fan would be the primary source of noise, this component had to operate quietly. A Joy Centrifoil Fan, size 20, with airfoil blades, is used because it has the lowest operating noise level of the fans considered.

Pitot tube utilized to determine air flow rate.—To determine the atmospheric concentrations of the materials collected, the volume of air sampled must be known. This is determined by integrating instantaneous sampling rates over the sampling time.

A Pitot tube and inclined manometer are used for measurement of the air velocity to determine the instantaneous air flow rate. This method was selected for two reasons: 1) It is a primary flow indicator and, therefore, does not require calibration against a more fundamental method and 2) it does not constrict the air flow.

Air flow path through system.—Air flows through the system in this sequence: filter→flow-measuring section→fan. A photograph of this arrangement is shown in figure 13 and illustrated schematically in figure 14. The filter is placed ahead of the other components for the following reasons:

> Least chance for contamination by fibers from duct insulation, oil from fan bearings, etc. This arrangement also eliminates possible hangup of airborne dirt particles in the system ahead of the filter
> Best arrangement for uniform flow through the filter
> Most convenient position for replacing filter units

Air enters the sampling system through an opening in a rear door of the truck and passes through the filter. A sponge rubber gasket seals the filter frame in the duct. The 30 × 24 in. duct cross section at the filter converges to a uniform 22 in. diameter round section, which continues straight for 42 in. to the fan inlet. The air flow rate through the system is measured at approximately the midpoint along the round section, by use of the Pitot tube located at the center line of the duct.

The centrifugal fan turns the air stream 90 degrees and discharges it through the roof via a 17⅝ × 19⅝ in. rectangular duct. An elbow mounted on the truck roof turns the air so that it discharges toward the front of the truck. Thus, the air is discharged 10 ft downstream from the point of intake and at a 5 ft higher elevation. During a sampling operation the truck is parked so that the air is discharged in the same general direction as the wind to eliminate recirculation.

Fan is driven by electric motor.—Two reasons prescribe the use of an electric motor, as opposed to an internal combustion engine, to drive the fan: 1) absence of by-product particulate matter in the case of the electric motor and 2) lower noise level of the electric motor.

The possibility of gaining independence from the local electric power supply by use of a gasoline motor-driven electric generator parked at a considerable distance from the air sampling unit was rejected, mainly on the basis of noise considerations but also partly because of possible contamination of the air being sampled.

The fan is driven by a 220 volt, 3-phase, 7½ hp motor. Power for the motor and other electrical equipment is supplied by a local utility company. Approximately 500 ft of 4 wire, heavy duty, electrical cable, in lengths of about 75 ft, allows flexibility in parking the truck. The cable has water-tight connectors.

Three-phase current at 208 to 240 volts or 440 to 480 volts can be accepted and transformed to the required 220 volts for the motor. The motor controls include the use of a reduced voltage starter to prevent overloading the local circuit during startup. Single-phase current at 115 volts, obtained from one of the output windings of the transformer, is utilized for lighting, instrumentation, and other accessories.

Air sampling equipment is mounted in Step-Van truck.—The vehicle in which the air sampling equipment is operated was selected after the approximate weight and dimensions of the apparatus had been determined. The truck has a Step-Van body with cab-over-engine arrangement, a style also popular with other air sampling investigators (*29*). A photograph of the vehicle is shown in figure 15.

Sampling equipment is removable from truck.—To facilitate the removal of equipment from the truck, the fan, motor, transformer, and motor controls were mounted on a wooden pallet so that they are removable as a unit. The loaded pallet, shown in figure 16, weighs about 1000 lb and can be handled with a fork lift truck. The ducting is fairly light and can be easily removed and transported in sections.

Sound is attenuated.—The sound developed by the apparatus and transmitted to the surrounding area is kept at a low level by the following means:

Flexible couplings at the fan
Independent rubber-in-shear mountings for the fan and motor
Sound-absorbing lining in the ducting
Insulation in truck body walls and roof
Partition between truck load space and driver's compartment
Wood floor over steel subfloor in the truck load space

Calibration and Operation of Equipment

Velocity profiles made to calibrate air flow.—Air flow calibrations were carried out by making 3 Pitot tube traverses across the circular duct section. The traversing positions were horizontal, vertical, and at 45°. The 3 traverses take into account the nonuniformities in flow that develop in the transition from the rectangular duct section to the circular section. Velocity profiles were plotted from which average velocities were determined. The average values obtained from each of the traverses were combined to obtain an over-all average air velocity.

Air flow calibration data are summarized in table 2. It is evident that a nonuniformity in flow does exist and that the results obtained from averages of the 3 traverses are necessary to establish calibration. A fixed relationship exists between the average velocity and the center line velocity for each traversing position. With the use of this proportionality, only the center line velocity has to be measured to obtain the over-all average velocity. Multiplying the latter value by the cross sectional area of the duct (2.64 ft^2) gives the instantaneous volumetric air flow rate.

TABLE 2.—Air flow calibrations

		Pitot tube position						
		Vertical		Horizontal		45°		Average
V_{avg}	fpm	1, 835	1, 865	1, 950	2, 000	1, 985	1, 985	1, 935
V_{cl}	fpm	2, 115	2, 165	2, 090	2, 145	2, 125	2, 110	2, 125
V_{avg}/V_{cl}		0. 867	0. 862	0. 933	0. 933	0. 934	0. 940	0. 911
Q	cfm	4, 840	4, 930	5, 150	5, 285	5, 240	5, 240	5, 115

Preliminary sampling was carried out mainly to check the operation of the equipment and observe the changes in air flow rate and filter resistance occurring in sampling periods which were longer than the anticipated 10 hours. Results obtained with two filters are shown in table 3.

Table 3.—Preliminary sampling data

Filter (in.)	Sampling time (hr)	Total flow (ft³)	Pressure drop Inches of water		Flow rate (cfm)		
			Start	Finish	Maximum	Average	Minimum
24 × 30	63	18, 424, 000	4. 20	4. 40	5, 230	5, 160	5, 040
24 × 24	151	38, 944, 000	5. 35	5. 85	4, 450	4, 280	4, 060

The data indicate very little change in flow rate even over relatively long sampling periods. The variations are due to the increase in filter resistance and changes in ambient temperature and barometric pressure. The minimum and maximum flow rates for the filter used for 63 hours were 97.6 and 101.3 percent of the average. For the second filter the range was from 95.1 to 104.1 percent. These variations are relatively low so that samples collected should be representative of the material in the atmosphere.

Noise is effectively attenuated.—For evaluation of the effectiveness of the sound-attenuating measures, sound-level measurements, with and without the equipment operating, were made with a Hermon Hosmer Scott, Type 410-B, Sound Level Meter, utililizing the Flat C scale. The data are shown in table 4.

Table 4.—Sound-level measurements*

Condition	10 Feet			At fan	At driver's seat
	Front of truck	Rear of filter	Middle of right side of truck		
Ambient Not operating All doors shut	62	62	63	53	59
Operating All doors open	68	74	69	88	83
Operating Truck doors shut Partition open	68	74	69	87	86
Operating Truck doors shut Partition shut	67	73	68	88	84

*All readings in decibels.

The sound level with the equipment not operating was 10 decibels lower inside the truck load space than outside, an indication of the attenuation effectiveness of the truck body. With the fan in operation, sound levels at the measuring points 10 ft from the vehicle increased by 5 to

12 decibels over ambient, but were still 13 to 21 decibels less than that at the fan. Experience has shown that one must be closer than 10 ft and directly in front of the air intake to notice any appreciable noise.

Tar is extracted from filter.—Recovery of the benzene-soluble tar from the filter is carried out in 3 steps:

Removal of the filter paper from the unit frame
Extraction of the benzene-soluble material from the filter paper
Concentration of the extracted material to obtain a tar

The filter paper is removed from the unit after 2 frame sides have been cut off on a large band saw. Approximately 99 percent of the filter paper is removed. The aluminum separators are displaced from the filter paper and washed in the extraction apparatus to recover any soluble material. The paper is cut into 3 portions, each of which is weighed:

(*a*) One complete pleat, approximately 3 ft², is used for lead determination
(*b*) Approximately one third of a pleat is used for inspection
(*c*) The remainder is extracted with a solution of 80 percent benzene and 20 percent methanol

The extraction operation is carried out in the 17 liter steam-heated stainless steel Soxhlet apparatus shown in figure 9. The paper is extracted for 48 hours, during which time approximately 32 syphoning actions occur.

The extract solution is drained into a distillation flask and distilled to about 1 liter. This solution is filtered, then concentrated to a tar by evaporation under vacuum as shown in figure 10. Final evaporation conditions are 0.1 mm Hg and 35° C.

The amount of tar collected in a sampling operation is determined by a correction of the tar weight in the evaporation flask for the following:

The filter paper left in the frame (~1%)
The filter paper used for other tests (1–2%)
The benzene-soluble impurities originally present in the filter paper (1.43 mg per ft²

Carbon Monoxide Level, Index of Auto Traffic

Atmospheric carbon monoxide and lead measured.—It has been shown that correlations exist between traffic density and atmospheric concentrations of carbon monoxide and lead (*30, 31*). Atmospheric concentrations of these two materials are measured in conjunction with the collection of benzene-soluble organic matter. The concentrations measured are used to estimate the atmospheric dilution of automobile exhaust gas, which, in turn, is to be used for estimation of the contribution by automobiles of carcinogenic material to the atmosphere, according to both the direct and indirect techniques.

A continuous measurement of carbon monoxide concentration is obtained with the use of a Beckman Model 21 nondispersive infrared analyzer having a sample cell length of 35 in. The instrument, mounted in the air sampling truck, is shown in figure 17. The analyzer casing has been modified to permit easier access to the cells and electronic components. The sample gas is dried with molecular sieve to remove water that would otherwise introduce a false and erratic response. Full scale response of 25 ppm carbon monoxide can be easily obtained by maintaining the sample pressure above 20 lb per in.^{2} gauge.

Proposed air sampling program.—The proposed air sampling program includes the collection of particulate matter in 3 cities: Detroit, New York, and Los Angeles. Detroit was chosen for preliminary sampling operations primarily to gain experience while the equipment was close to the Research Laboratories. Chemical studies with atmospheric tar collected in Detroit are in progress.

Sampling operations in New York City and Los Angeles will be carried out at several locations in each city at sites with different traffic conditions.

CONCLUSIONS

A mobile laboratory for collecting particulate matter from the atmosphere on an "absolute" filter at an air flow rate of 5000 cfm has been built. Preliminary operations have shown that the apparatus meets all of its design criteria. It is expected to provide a useful facility in the study of the relationship between automobile effluents and potentially carcinogenic material in the atmosphere.

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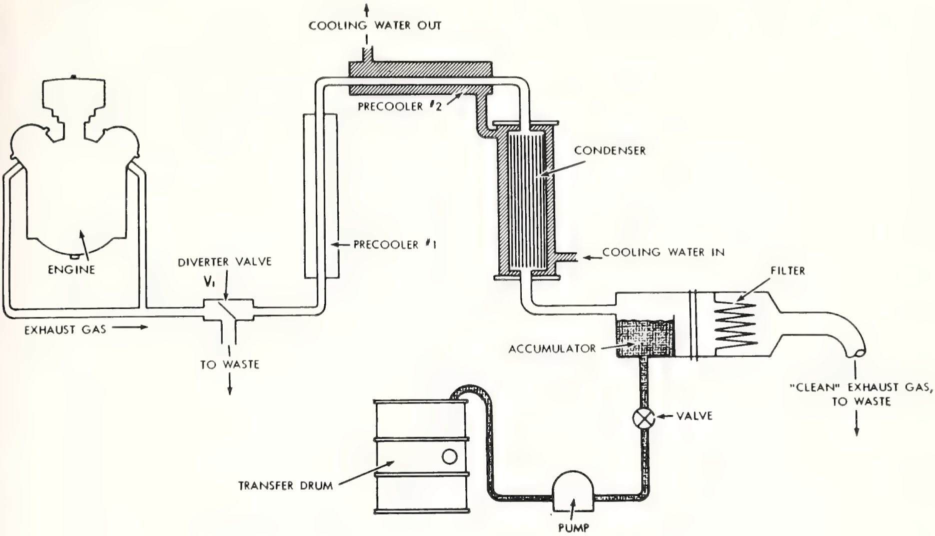
PLATES

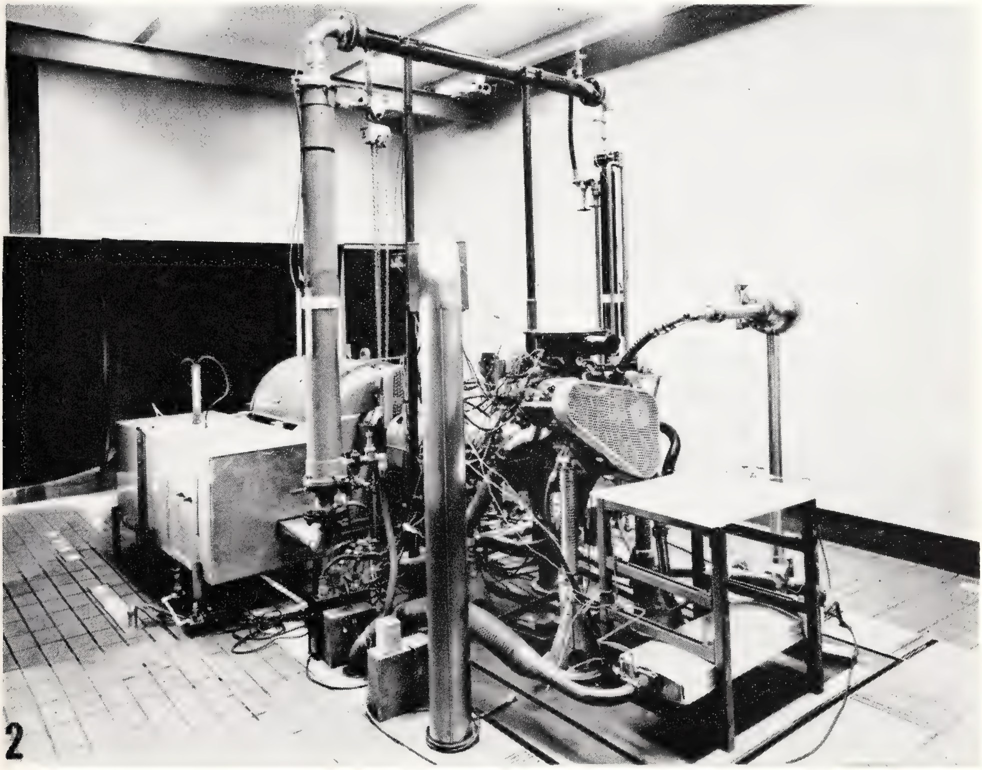
PLATE 1

FIGURE 1.—Combined condensation-filtration apparatus for collection of particulate matter in exhaust gas.

FIGURE 2.—Engine and complete tar collection system.


COOLING WATER OUT
PRECOOLER #2
CONDENSER
PRECOOLER #1
COOLING WATER IN
ENGINE
DIVERTER VALVE
V₁
FILTER
EXHAUST GAS
TO WASTE
ACCUMULATOR
"CLEAN" EXHAUST GAS, TO WASTE
VALVE
TRANSFER DRUM
PUMP

1

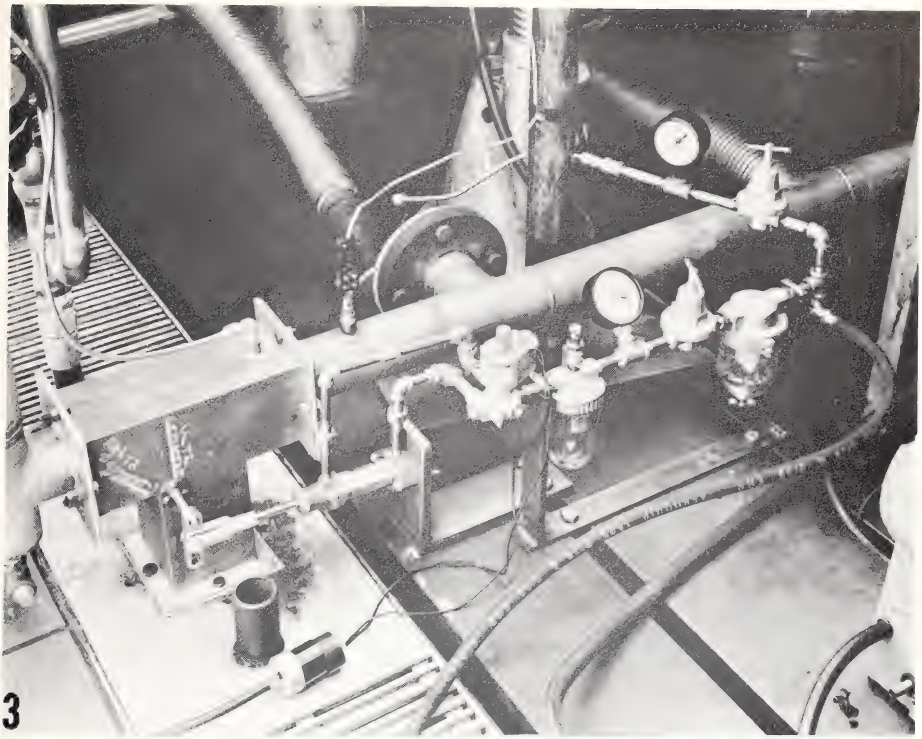


2

Plate 2

Figure 3.—Exhaust line T- valve.

Figure 4.—Exhaust filter unit.



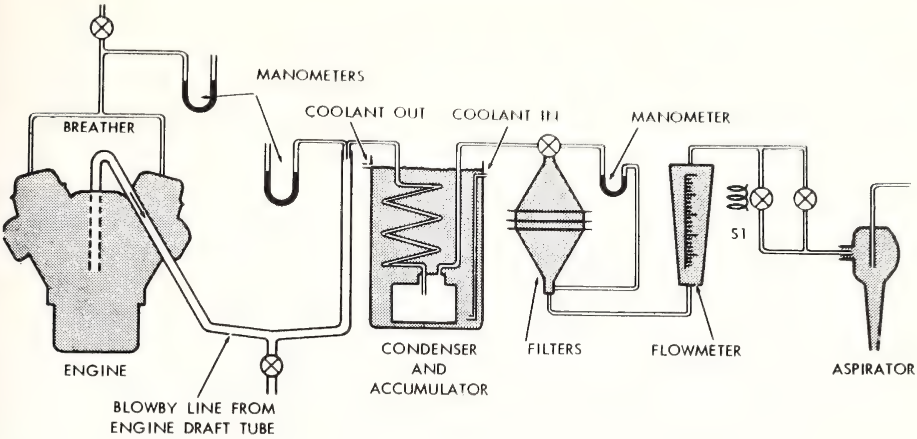
3

4

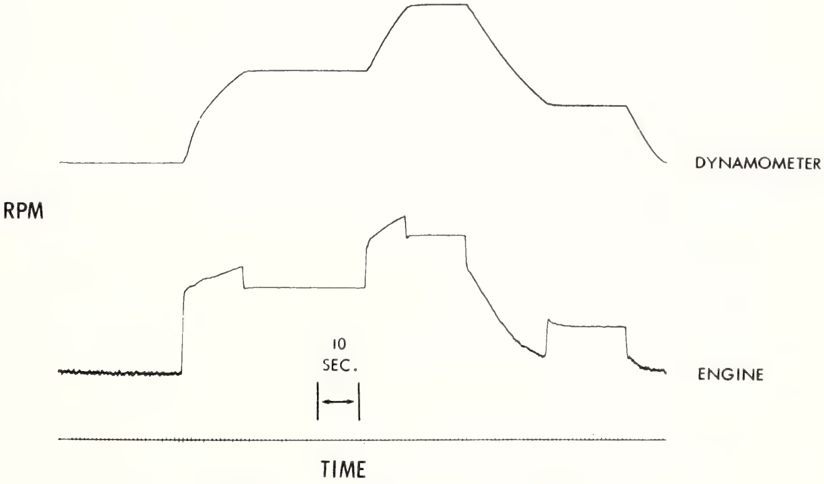
Plate 3

Figure 5.—Apparatus for recovery of tar from blowby.

Figure 6.—Recording of engine and dynamometer rpm for a typical cycle.


MANOMETERS
BREATHER
COOLANT OUT
COOLANT IN
MANOMETER
S1
ENGINE
BLOWBY LINE FROM ENGINE DRAFT TUBE
CONDENSER AND ACCUMULATOR
FILTERS
FLOWMETER
ASPIRATOR

5

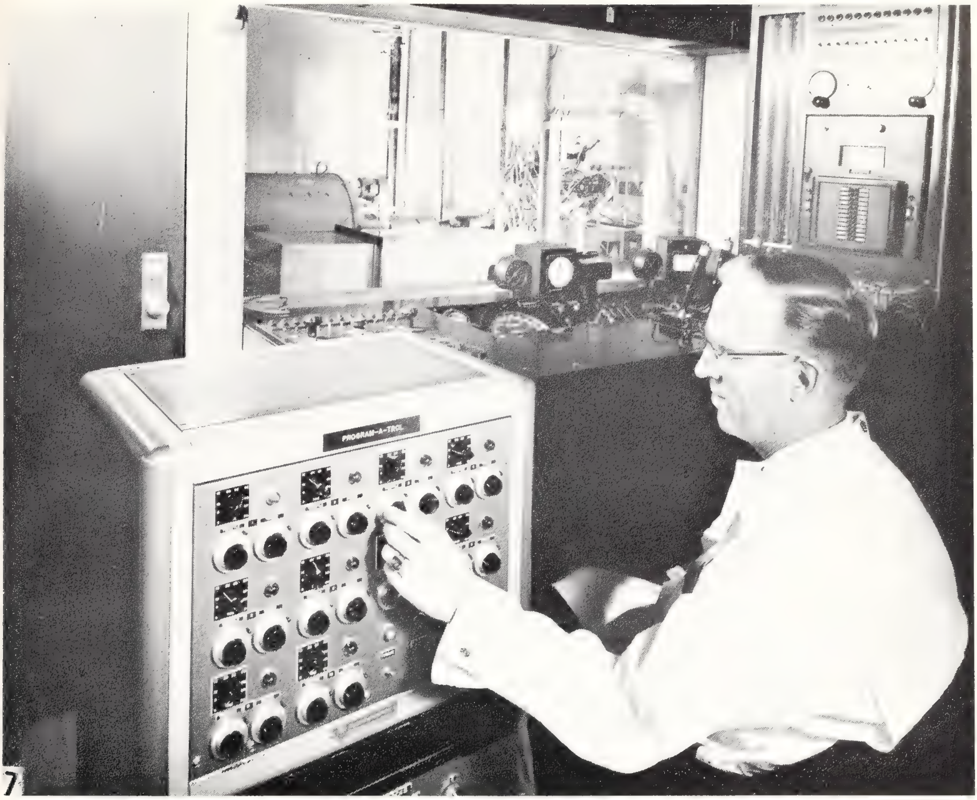


6

DYNAMOMETER IS DRIVEN THROUGH A TORQUE CONVERTER TRANSMISSION

Plate 4

Figure 7.—Engine controls showing the Program-O-Trol.


PROGRAM-A-TROL

7

PLATE 5

FIGURE 8.—Countercurrent liquid-liquid extraction column.

YORK-SCHEIBEL EXTRACTION APPARATUS USED TO RECOVER TAR FROM CONDENSED WATER PHASE

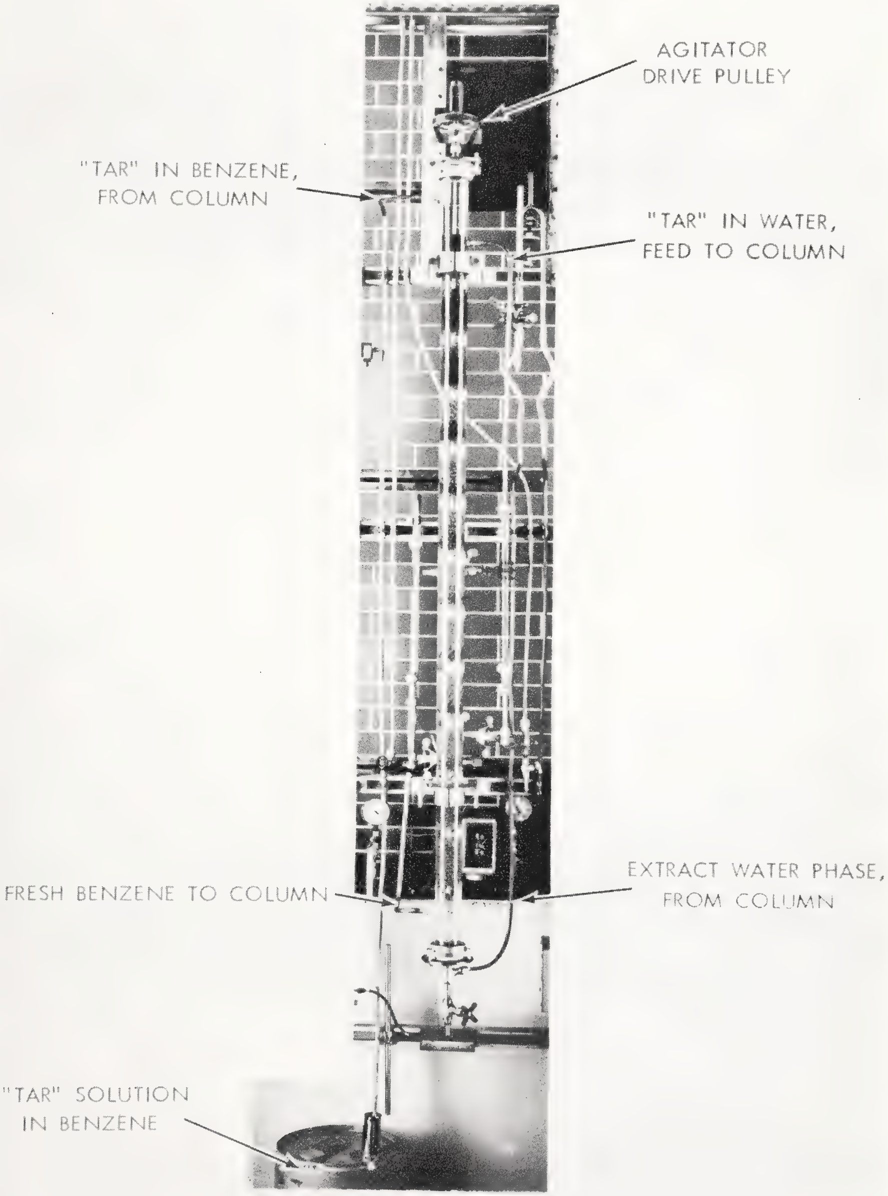
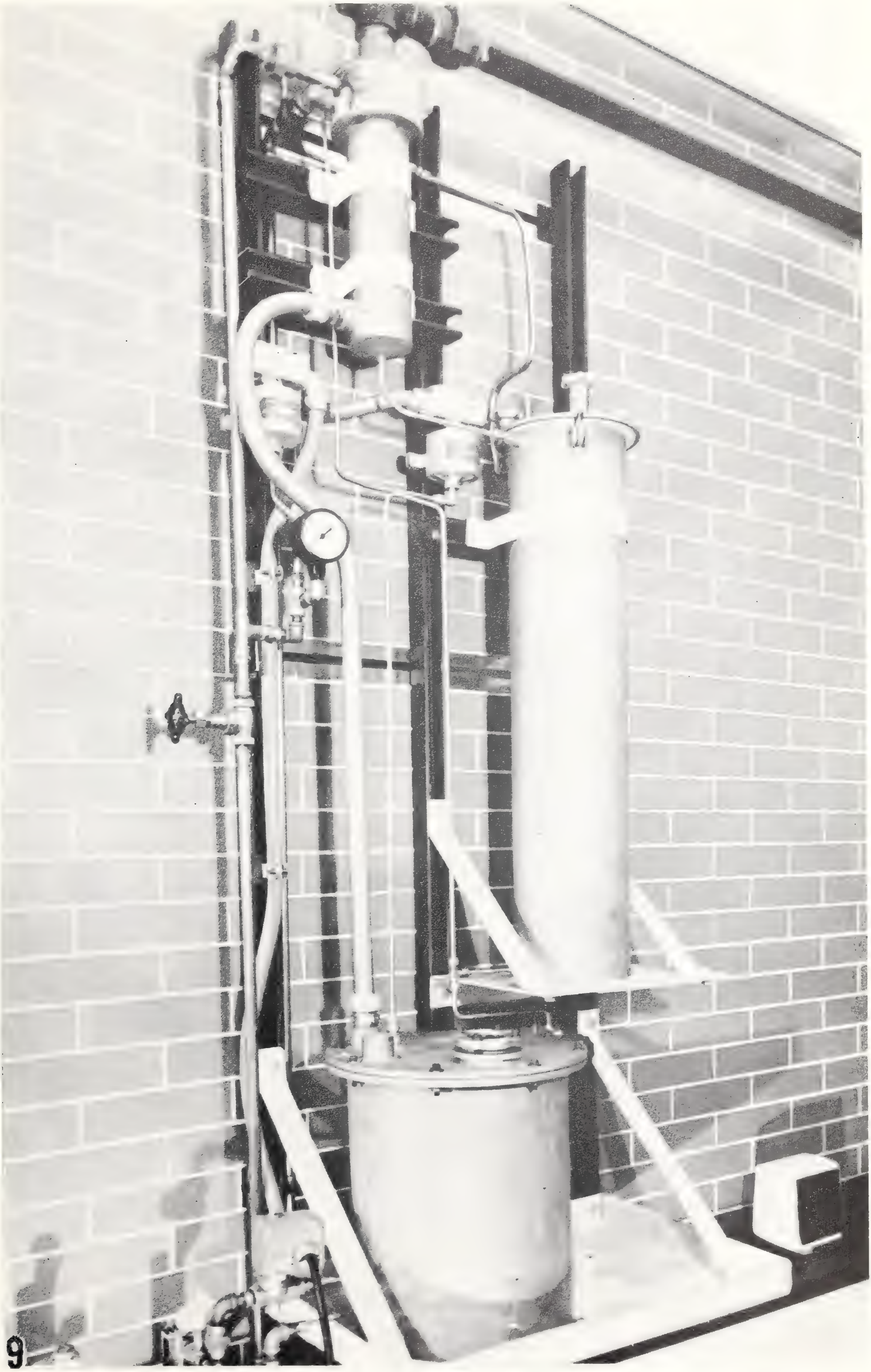

AGITATOR DRIVE PULLEY
"TAR" IN BENZENE, FROM COLUMN
"TAR" IN WATER, FEED TO COLUMN
FRESH BENZENE TO COLUMN
EXTRACT WATER PHASE, FROM COLUMN
"TAR" SOLUTION IN BENZENE

Plate 6

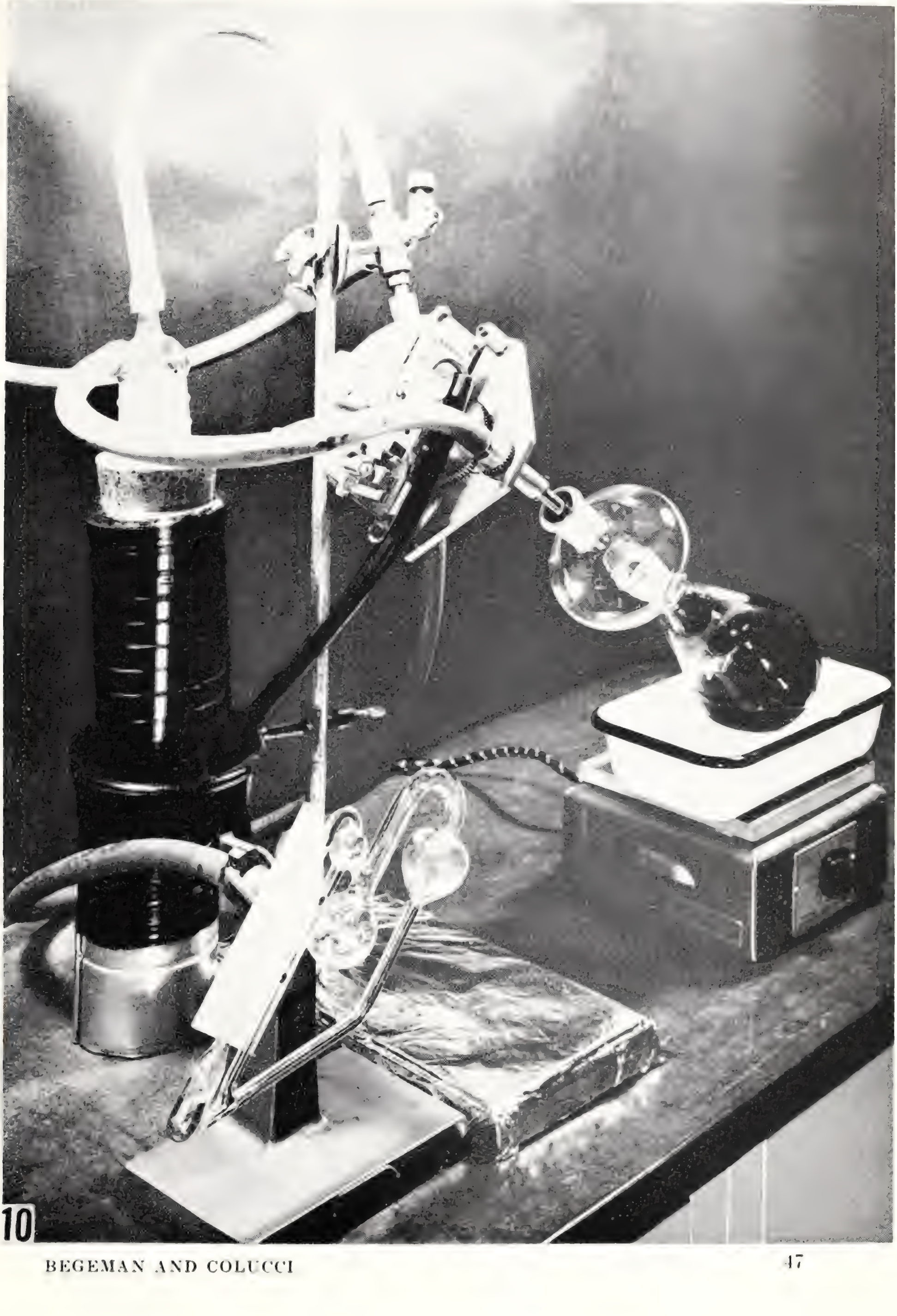
Figure 9.—Soxhlet apparatus for extraction of filters.



9

Plate 7

Figure 10.—Flask oscillating device used in removal of last traces of solvent from tar.

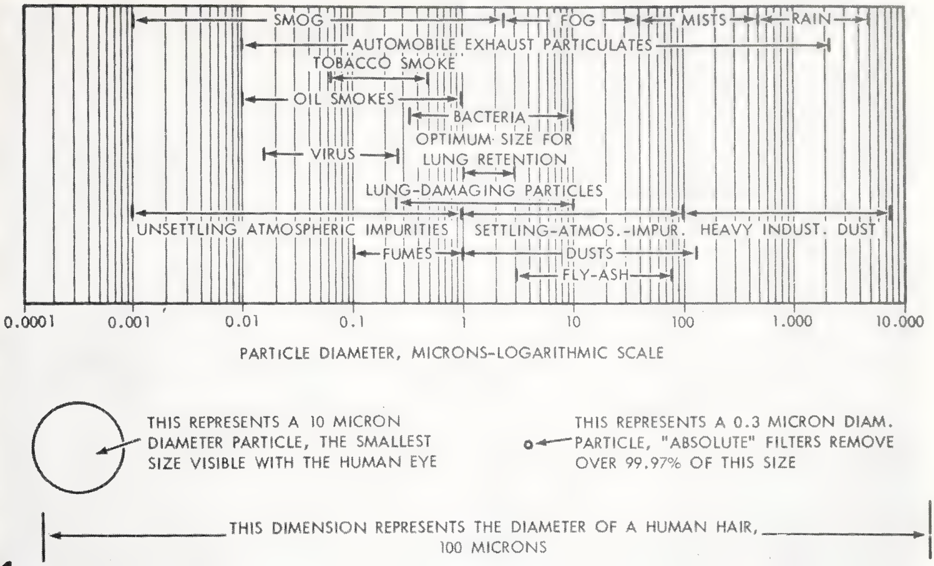


10

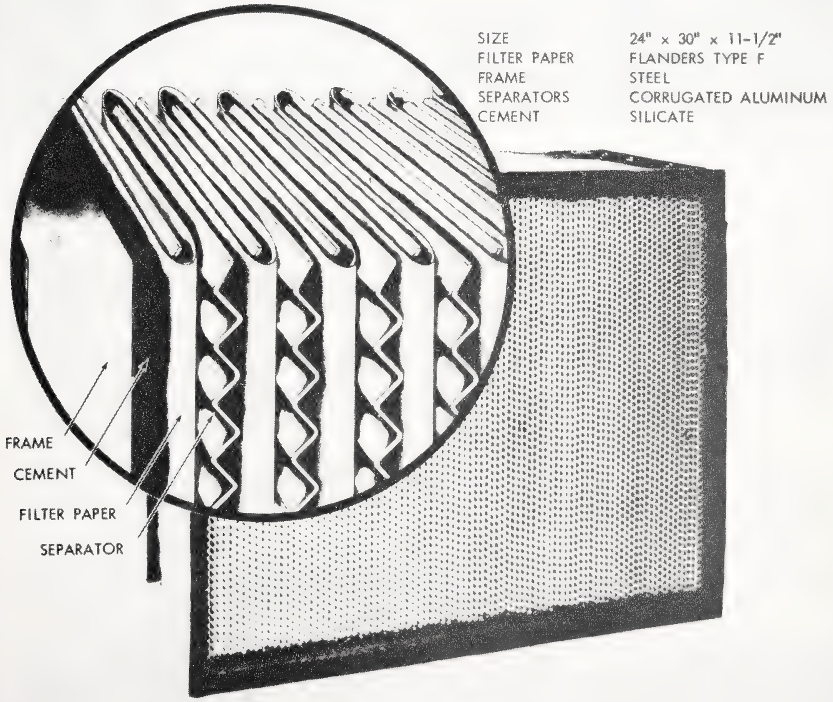
PLATE 8

FIGURE 11.—Size ranges of particles found in the atmosphere.

FIGURE 12.—Typical "absolute" filter unit construction.



11


FRAME
CEMENT
FILTER PAPER
SEPARATOR

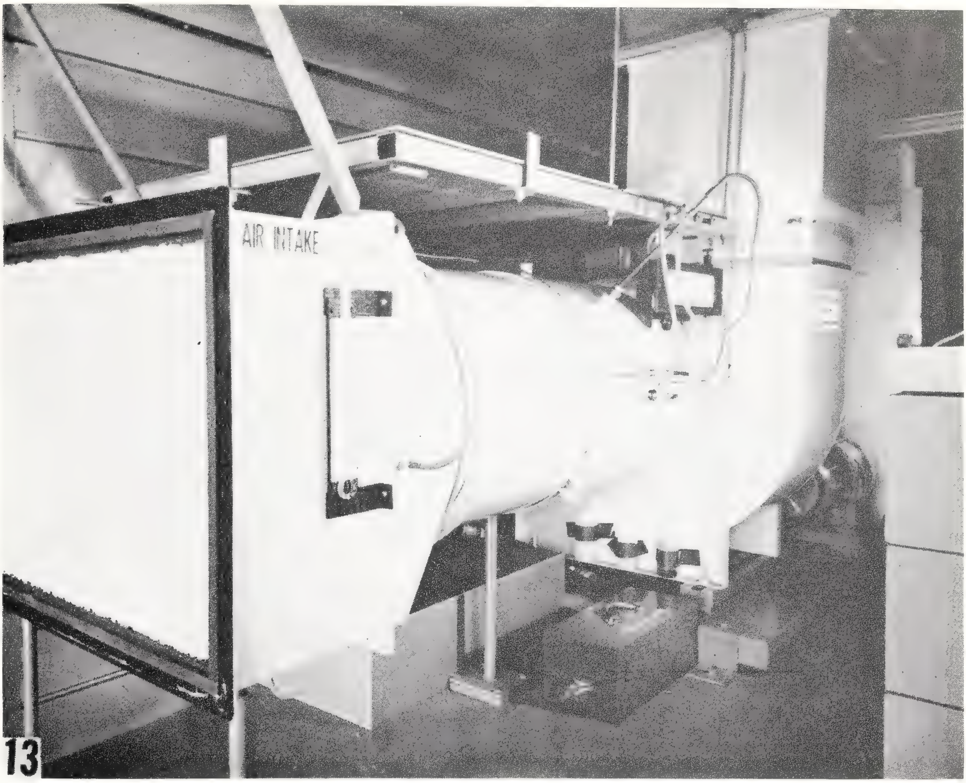
SIZE	24" x 30" x 11-1/2"
FILTER PAPER	FLANDERS TYPE F
FRAME	STEEL
SEPARATORS	CORRUGATED ALUMINUM
CEMENT	SILICATE

12

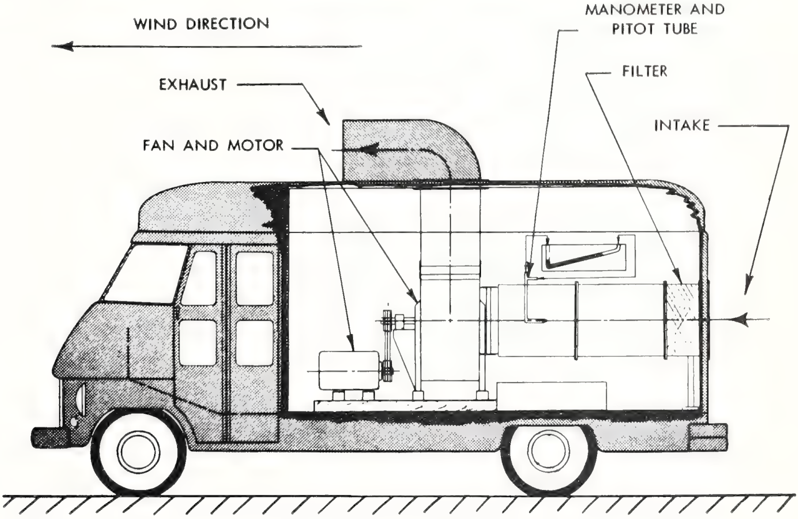
Plate 9

Figure 13.—Particulates sampling system.

Figure 14.—Schematic drawing of air sampling system.


AIR INTAKE

13


WIND DIRECTION
EXHAUST
FAN AND MOTOR
MANOMETER AND PITOT TUBE
FILTER
INTAKE

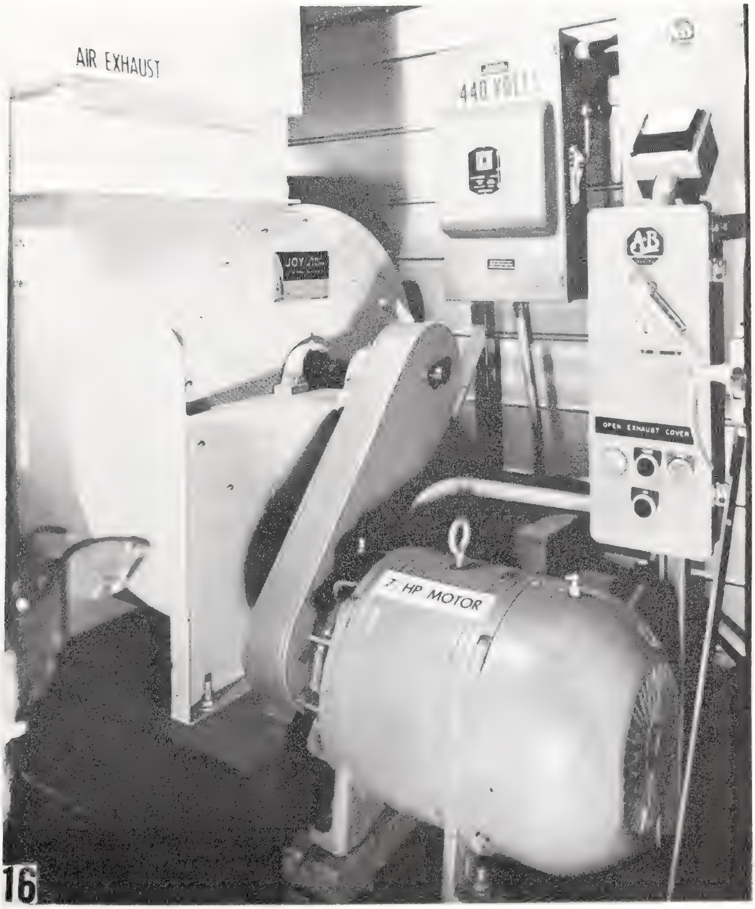
14

Plate 10

Figure 15.—Photograph of air sampling vehicle.

Figure 16.—Pallet mounting of fan, motor, transformer, and motor controls.

15


AIR EXHAUST
440 VOLT
JOY
A-B
OPEN EXHAUST COVER
7 HP MOTOR

16

Plate 11

Figure 17.—Infrared analyzer for determination of carbon monoxide.

BEGEMAN AND COLUCCI

DISCUSSION

MR. VON LEHMDEN: I would like to ask Mr. Begeman if he could explain why he chose a mixture of benzene and methanol for extracting the filter.

MR. BEGEMAN: I refer the question to Dr. Hoffmann.

DR. HOFFMANN: We tried various solvents with the loaded filter and concluded that the best was benzene methanol in the proportion of four to one by volume.

DR. COMMINS: I would like to ask Mr. Begeman at what temperature is his collecting filter and whether he attempted to assess what percentage of material went through the filter, if any.

MR. BEGEMAN: The temperature of the filter was below room temperature, that is, below about 70° F. We investigated efficiency of collection in a small-scale experiment. We found that the #50 FG medium recovered a minimum of 96 percent of that which was stopped on an absolute filter. We did not determine whether there was any loss of benzo[*a*]pyrene or other compounds.

MR. HOWERTON: Did you plot the velocity in the center on axis versus the off axis velocity?

MR. COLUCCI: The velocity profile consisted of making traverses across the duct section.

MR. HOWERTON: Across horizontal and vertical?

MR. COLUCCI: Yes.

MR. HOWERTON: And you got a curve. Is it a parabolic curve?

MR. COLUCCI: Yes, fairly parabolic.

MR. HOWERTON: Both ways?

MR. COLUCCI: Yes. There are differences between the two that are caused by the transition from the rectangular section to the circular section.

MR. HOWERTON: But that is minor?

MR. COLUCCI: Yes, small, very small.

MR. HOWERTON: Could you give me the velocity profile? I did not understand it from your slide. At, say, 1 in. from the edge of the tube, what would be the ratio of the velocity at the center?

MR. COLUCCI: The velocity goes up very fast as you go away from the duct wall. It increases quite rapidly to almost the average velocity when you are probably an inch or two away from the wall.

MR. HOWERTON: But you do not have exact curves on that?

MR. COLUCCI: I have curves but I do not have them here. I could get them to you if you would like.

MR. TABOR: One of the problems we have is the collection of a large sample that can be aliquoted for interlaboratory comparisons. Is the deposit on the filter sufficiently uniform that it may be used for this purpose?

MR. COLUCCI: Well, the easiest way to get an aliquot is after the extraction of the sample.

MR. TABOR: Well, I am thinking of the original particulate matter itself.

MR. COLUCCI: You can do this by just removing a complete pleat.

MR. TABOR: Is this uniform?

MR. COLUCCI: Yes, from one pleat to the next they are identical; however, along the pleat from the front of the filter to the rear there is some difference. Most of the deposition is at the rear of the pleat.

MR. STERN: I would like to comment on the statement that was made that there is a 50 percent variation to be expected in National Air Sampling Network flow rates. This is not the norm, and I do not want the impression to be retained that it is. It would be quite abnormal for this to occur. Where we expect very heavy filter samples that would tend to cause greatest flow reduction, we use a flow-regulating device to smooth out flow.

MR. COLUCCI: My data on this were obtained from the original publication from the National Air Sampling Network, which covered data from 1953 to 1957. This publication indicated that some of the samples obtained had this great discrepancy between flow rates, but it did not say that they all did, and I hope I did not imply this.

DR. HOFFMANN: Mr. Colucci mentioned that our group asked for 1 g of organic extractable matter from the filter for chemical analysis. This is not for chemical analysis alone; this is also for biological testing. We do not need 1 g for a chemical analysis.

MR. COLUCCI: This is another reason that we have made the apparatus as large as it is, so that we can also obtain samples from the atmosphere for use in biological experiments.

Developmental Work on Bioassay Technique for Atmospheric Pollutants [1]

W. D. WON *and* J. F. THOMAS,[2] *Sanitary Engineering Research Laboratory, College of Engineering and School of Public Health, University of California, Berkeley, California*

SUMMARY

Numerous compounds have been separated in pure form from polluted atmospheres by chromatography and other advanced analytical techniques. Although many of these compounds had not been identified, it was desirable to assay for intracellular activities because of known adverse physiological effects of air pollutants on plants and animals. Striking morphological alterations were noted in heart infusion broth cultures of *Bacillus megaterium* supplemented with benzo[*a*]pyrene and 3-methylcholanthrene. These anomalies were not apparent in cultures supplemented with noncarcinogenic hydrocarbons such as phenanthrene and anthracene. The addition of hydrocarbons to the heart infusion broth medium did not appreciably affect the growth rate of the organisms. Biochemical examinations of *B. megaterium* were made with cultures grown in a simple, chemically defined medium that contained glucose as the sole energy source. Morphological alterations did not develop in these cultures supplemented with carcinogenic hydrocarbons, but a growth suppression was noted. Other significant findings concerned lipide synthesis, aerobic glycolysis, and infrared spectra of lipide material.—NCI Monogr. No. 9: 59–69, 1962.

NUMEROUS ORGANIC compounds have been separated in relatively pure form from polluted atmospheres, but only a small percentage has been identified. The identified compounds include several powerful carcinogens; for this reason it is desirable to assay the unidentified compounds for carcinogenic activity. Conventional assay techniques are not feasible, as only minute quantities of material are available. A bioassay technique that uses bacterial colonies in place of conventional laboratory animals has been developed; the method is sensitive to small quantities of test material. The new technique appears promising for a number of reasons: 1) Minute quantities of test material, added as a medium supplement, are adequate; 2) a large number of subjects is readily available; 3) all tests are relatively rapid; 4) the use of single cells makes cell response easily identifiable; and 5) the environment of the test organism is more easily controlled. The resulting growth, cell morphology, and biochemical modifications of the microorganisms are observed and used as indicators of activity.

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[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29-31. 1961.

[2] Assistant Research Bacteriologist and Associate Professor of Sanitary Chemistry.

A limited search of the literature revealed that effects of chemical carcinogens on microorganisms have been reported. Goldstein (*1*) first reported a growth-accelerating effect on bacterial cultures exerted by chemical carcinogens. By comparing the cell yields of *Escherichia communior* grown in media containing either dibenz[*a,h*]anthracene or methylcholanthrene (carcinogens) with those grown in media containing phenanthrene (a noncarcinogen) and in controls, Goldstein obtained 50 percent greater yields from the carcinogen-containing cultures than from the noncarcinogen-containing and the control cultures in a short period of incubation (9 hours).

Hopper and Clapp (*2*) extended and confirmed Goldstein's observations, using similar organisms in analogous studies. Moreover, these workers presented evidence correlating the effects on bacterial reproduction of carcinogenicity of compounds and degree of stimulation.

Novelli (*3*) described experiments with different generic types, and indicated that *Staphylococcus aureus* was the organism most responsive to the stimulatory action of chemical carcinogens.

Alterations in cell morphology elicited by chemical carcinogens have also been described. Dodge and Dodge (*4*) observed giant-cell formations in *Saccharomyces ellipsoideus* cultures to which methylcholanthrene was added. Outstanding abnormal morphological changes, including the occurrence of "Siamese twins," triplets, and other varied shapes, were also seen in paramecium cultures supplemented with benzo[*a*]pyrene (*5*).

Contradictory and discordant reports were also found in the literature. Chemical carcinogens effecting growth inhibition were described by Miller *et al.* (*6*), Reese and Reese (*7*), and Brown (*8*); and a description of negative effects of chemical carcinogens on bacterial cells was given by Melroy and Spencer (*9*).

No reference pertaining to the chemical and biochemical changes associated with affected cells was found in the literature. However, because morphological alterations have occurred in *Bacillus megaterium* cultures grown in the presence of a carcinogen, attention was directed toward isolation and comparison of cell constituents between these and normal cells.

EXPERIMENTAL CONDITIONS

Stock bacterial slants including *Escherichia coli*, *Bacillus megaterium*, *Pseudomonas fluorescens*, and *Serratia marcesens* were grown on blood-agar-base-medium (Difco) for 24 hours at 30° C and stored at 5° C. At 2-month intervals, these were subcultured on freshly prepared agar slants.

The media used throughout the study were Difco Heart Infusion Broth (HIB) and a chemically defined medium (CD) of the following composition, in g per liter: $Na_2HPO_4 \cdot 12H_2O$, 15.0; KH_2PO_4, 3.0; NaCl, 1.0; NH_4Cl, 1.0; and $MgSO_4 \cdot 7H_2O$, 0.2. Media were dispensed in 200 ml quantities into 400 ml Erlenmeyer flasks. Sterilization was effected by saturated steam at 15 lb per in.^{2} for 15 minutes. After the CD medium had cooled, sterile glucose was added aseptically to produce a 0.5 percent concentration of the carbon source with a *p*H of 7.2

Benzo[*a*]pyrene, 3-methylcholanthrene, phenanthrene, and anthracene were the hydrocarbons used for developmental work, as they have known carcinogenic activity and are associated with the condensed polynuclear aromatic hydrocarbon type of atmospheric pollutants. The standard concentration for the hydrocarbons was 0.025 μg per ml based on the limiting concentration of the least soluble test compound, 3-methylcholanthrene. We prepared stock solutions by dissolving the hydrocarbon in 30 ml of methylene chloride made up to 100 ml with 95 percent ethanol.

A standard inoculum was prepared by transfer of a single loop of the stock culture to 30 ml HIB or CD. The inoculum was incubated statically for 24 hours at 30° C. One ml of culture was then transferred to 100 ml portions of fresh medium supplemented with a carcinogen, or with a noncarcinogen, and contained in 500 ml Erlenmeyer flasks. The cultures were incubated at 30 to 32° C on a Brunswick gyratory shaker. At specified intervals, these cultures were examined for morphological changes, viable yields, and biochemical changes.

ANALYTICAL METHODS

For microscopic examination of morphological variations, standard smears, made at specified intervals, were stained with Giemsa stain. A parallel series of smears was also prepared for the examination of internal structures according to the technique suggested by Robinow (*10*).

For lipide-fraction characterization, 100 ml aliquots of culture containing approximately 1.0×10^{10} viable cells per ml were sedimented by centrifugation at $3000 \times g$ for 20 minutes. The washed, harvested cells were then extracted with methylalmethanol and dried in a stream of nitrogen at 60° C. A portion of the dried extracts was dissolved in CCl_4 and spectra were obtained with a Beckman Model IR-5 spectrophotometer.

An alternative technique was to smear and air-dry a sample on the surfaces of KBr discs that were tilted slightly so as to produce wedge-shaped films of variable thickness. Such a procedure permitted structural analysis with very small samples, and the variable thickness of the film permitted the regulation of the sample beam to give identical percent transmission with a common band absorption at a specified wavelength.

For the methylene blue reduction test, 72-hour shaken cultures were washed twice with normal saline solution by centrifugation at $3000 \times g$ for

20 minutes. The cells were resuspended in saline solution and standardized to approximately 1.0 × 10^{10} viable cells per ml in terms of optical density on a Bausch and Lomb Spectronic 20 colorimeter. Aliquots, 9 ml, of cell suspension were then transferred to 150 × 18 mm pyrex tubes, and 1.0 ml of 1/20,000 methylene blue solution was added. The tubes were fitted with rubber stoppers, mixed gently, and incubated at 37° C. Visual judgment of the complete reduction of the dye was taken as the endpoint.

Lactic acid concentration was determined colorimetrically at 560 mμ on a Beckman Model B Spectrophotometer according to the method of Barker (*11*).

EXPERIMENTAL RESULTS

For the study of the effect of hydrocarbons on the growth of *E. coli*, *B megaterium*, *P. fluorescens*, and *S. marcesens*, the HIB and CD media were used. Data presented in table 1 represent the growth yields of the various organisms grown in the CD medium. The results were the average counts of triplicate plates at specified periods. It can be seen that in the first 6 hours of incubation a pronounced growth suppression occurred in the CD cultures which contained a carcinogenic hydrocarbon (3-methylcholanthrene or benzo[*a*]pyrene), while those which contained a noncarcinogen (phenanthrene or anthracene) exhibited no effect. The growth suppression, however, varied with the organisms studied. By the 24th hour, the inhibitory effect exerted by the carcinogens was no longer apparent. It is interesting also that the presence of these hydrocarbons did not affect an accelerated death rate in the cultures.

While the growth rate was not appreciably affected in the HIB series of cultures (table 2), large cells were produced in those that were supplemented with carcinogens. No effect was noted in the media supplemented with noncarcinogens. Again, despite the identical growth conditions, the yield of large cells varied with the organism studied. In no case, however, was there a cell population constituted homogeneously of large cells. In this respect, it was observed that *B. megaterium* was the most responsive organism, yielding the largest proportion of large cells after 24 hours of incubation. It was of particular interest that large cells were detectable in the first 7 hours of incubation and reached a plateau at 20 to 24 hours. However, continued incubation produced progressive degeneration of cells, such as shrinking and rounding, and also produced cells with obscure outlines. Marked pleomorphism occurred after 7 days of shaking at 37° C.

Microscopically, with the exception of filamentous forms in the *E. coli* and *S. marcesens* cultures, the large cells ranged from 50 to 100 percent longer and approximately 50 percent wider than the normal cells. While the normal and unaffected cells of *B. megaterium* stained evenly after 24 hours of growth, the cytoplasm of the large cells appeared reticular. Occasionally, large cells containing a large number of metachromatic granules were also noted. The gross cytologic picture remained the

TABLE 1.—Effect of polycyclic aromatic hydrocarbons on the viable yields of 4 species of bacteria grown in CD medium

Species	Supplement	Viable cells per ml					
		0 Hour ($\times 10^4$)	6 Hours ($\times 10^5$)	24 Hours ($\times 10^8$)	48 Hours ($\times 10^8$)	72 Hours ($\times 10^8$)	96 Hours ($\times 10^8$)
Escherichia coli	Control	1.0	22.0	8.0	8.7	8.0	7.5
	Phenanthrene	1.0	18.0	8.6	8.4	8.0	6.8
	Anthracene	1.0	20.0	8.0	8.2	8.1	7.2
	Benzo[*a*]pyrene	1.0	2.3	7.9	8.2	8.6	7.6
	3-Methylcholanthrene	1.0	3.1	8.3	8.3	8.2	7.2
Bacillus megaterium	Control	2.6	32.0	0.65	0.88	0.90	0.80
	Phenanthrene	2.6	36.0	0.72	0.86	0.85	0.84
	Anthracene	2.6	29.0	0.70	0.77	0.88	0.80
	Benzo[*a*]pyrene	2.6	1.4	0.60	0.87	0.88	0.78
	3-Methylcholanthrene	2.6	2.2	0.70	0.80	0.85	0.80
Pseudomonas fluorescens	Control	1.4	11.00	6.0	—	—	—
	Phenanthrene	1.4	13.00	5.0	—	—	—
	Anthracene	1.4	10.00	6.0	—	—	—
	Benzo[*a*]pyrene	1.4	0.72	6.6	—	—	—
	3-Methylcholanthrene	1.4	0.84	5.1	—	—	—
Serratia marcesens	Control	1.2	1.00	3.5	4.1	4.5	4.0
	Phenanthrene	1.2	1.10	3.0	4.3	5.0	3.8
	Anthracene	1.2	1.30	3.5	3.9	5.2	4.3
	Benzo[*a*]pyrene	1.2	0.74	3.5	4.1	6.0	4.5
	3-Methylcholanthrene	1.2	0.51	3.0	4.1	5.5	4.3

Table 2.—Effect of polycyclic aromatic hydrocarbons on the viable yields of 3 species of bacteria grown in HIB medium

Species	Supplement	0 Hour ($\times 10^4$)	6 Hours ($\times 10^6$)	24 Hours ($\times 10^9$)	48 Hours ($\times 10^9$)	72 Hours ($\times 10^9$)	96 Hours ($\times 10^9$)
Escherichia coli	Control	1. 0	4. 6	3. 1	6. 0	6. 8	5. 2
	Phenanthrene	1. 0	4. 4	3. 3	6. 0	6. 7	5. 3
	Anthracene	1. 0	4. 6	3. 2	6. 1	6. 6	5. 0
	Benzo[*a*]pyrene	1. 0	4. 6	3. 1	6. 0	6. 7	5. 2
	3-Methylcholanthrene	1. 0	4. 5	3. 2	6. 2	6. 8	5. 3
Bacillus megaterium	Control	2. 6	7. 2	5. 5	7. 6	8. 8	8. 0
	Phenanthrene	2. 6	7. 1	5. 6	7. 3	8. 6	8. 0
	Anthracene	2. 6	6. 9	5. 6	7. 7	8. 5	8. 1
	Benzo[*a*]pyrene	2. 6	7. 0	5. 5	7. 5	8. 7	8. 1
	3-Methylcholanthrene	2. 6	7. 0	5. 3	7. 6	8. 6	8. 1
Pseudomonas fluorescens	Control	1. 4	6. 7	5. 2	8. 0	8. 3	7. 8
	Phenanthrene	1. 4	6. 8	5. 2	8. 1	8. 2	7. 9
	Anthracene	1. 4	6. 5	5. 1	7. 9	8. 3	7. 8
	Benzo[*a*]pyrene	1. 4	6. 8	5. 3	7. 9	8. 0	7. 7
	3-Methylcholanthrene	1. 4	6. 6	5. 1	8. 2	8. 1	7. 8

same except for the display of incomplete septa and indentations on the cell walls when the smears were hydrolyzed with 0.1 N HCl at 60° C for 10 minutes before staining.

Since preliminary experiments showed that *B. megaterium* was the most responsive organism for this work, it was selected for subsequent biochemical studies. The CD medium was selected to maintain constant environmental conditions, since batch variations in commercial media have occurred.

The comparative reduction rate of methylene blue is illustrated by the data presented in table 3. The enzyme activity of *B. megaterium* was, in all cases, considerably enhanced with respect to the control preparation. Reduction time of the dye was progressively lowered as the carcinogenic potency of the compound increased. Accordingly, the reduction time of the preparation treated with 3-methylcholanthrene was $<$ that of the culture treated with benzo[*a*]pyrene $<$ anthracene-treated $<$ phenanthrene-treated $<$ control.

TABLE 3.—Effect of polycyclic aromatic hydrocarbons on the methylene blue reductase activity of *Bacillus megaterium* at 37° C

Supplement	Reduction time (min)		
	24-Hour growth	48-Hour growth	72-Hour growth
Control	50	90	65
Phenanthrene	40	45	40
Anthracene	35	45	40
Benzo[*a*]pyrene	25	40	35
3-Methylcholanthrene	20	35	25

Infrared spectra of the lipide material from cells grown in the presence of a carcinogen showed absorption peaks at 5.1 μ (1950 cm^{-1}), at 7.7 μ (1300 cm^{-1}), and at 10.6 μ (942 cm^{-1}). These peaks were consistently absent in the noncarcinogenic and control extracts. The spectra of the latter materials were remarkably similar.

Only the CD medium was used to study the influence of carcinogenic and noncarcinogenic hydrocarbons on the lipide and lactate productions of the organism. Data obtained from this study are presented in table 4.

TABLE 4.—Effects of arenes on lactate formation and lipide systhesis by *Bacillus megaterium* grown in defined medium

Supplement	Total lipide (mg/100 ml)			Lactic acid (μg/ml)		
	24 Hours	48 Hours	72 Hours	24 Hours	48 Hours	72 Hours
Control	7. 3	17. 4	19. 3	15. 0	32. 0	60. 0
Phenanthrene	8. 6	20. 3	25. 0	16. 0	32. 6	64. 0
Anthracene	9. 7	23. 3	26. 2	15. 8	34. 0	66. 0
Benzo[*a*]pyrene	10. 1	31. 0	29. 4	16. 6	39. 0	73. 0
3-Methylcholanthrene	10. 5	26. 0	27. 8	16. 4	38. 0	70. 0

As indicated by the results, there are quantitative differences in the total lipide and lactic acid production of cells grown in the presence of carcinogenic hydrocarbons compared to the control and to those grown in the presence of noncarcinogenic arenes.

DISCUSSION AND RESULTS

The effects of carcinogens on bacterial growth and morphology have been the subject of investigation by earlier workers. Unfortunately, the results revealed many contradictions and inconsistencies. Growth stimulation, as well as inhibition, for bacterial cultures due to the influence of polycyclic aromatic hydrocarbons have been reported (*1–3*, *6*, *9*).

Brown (*8*), who attributed the discordant results to the use of unstable suspensions or solutions of uncertain concentration, prepared and employed water-soluble derivatives of these hydrocarbons. Among the data that emerged from his report are the following: (*a*) Carcinogenic derivatives, though weaker in carcinogenicity than the parent compounds, were decidedly growth-inhibitory, whereas a noncarcinogenic derivative was ineffective; (*b*) the degree of growth inhibition varied with the organism studied; and (*c*) marked inhibition in one medium may be consistently absent in another.

The results of the present investigation on bacterial cultures grown in HIB and CD media supplemented with the natural forms of hydrocarbons were in exact agreement with Brown's findings; growth inhibition, which varied among species, occurred in the CD but not in the HIB medium. In morphological modifications, Brown observed no gross abnormalities, although previous workers described giant-cell production in microbial cultures supplemented with carcinogenic hydrocarbons (*4*, *5*, *7*).

In the present investigation, the induction of large cells in the HIB medium containing a carcinogen may be the result of the chemical *per se* or the combined action of the hydrocarbon and certain factors in this complex medium. It is also a possibility that formation of large cells was induced by the impurity of the hydrocarbon or by poor environmental conditions. Large cells readily reverted to their normal appearance when subcultured in a carcinogen-free medium. This is in contrast to the stable, large-celled strains of *Pasteurella pestis* (*12*) and *E. coli* (*13*), which are produced by exposure to the oxygenated derivatives of the terpene type of hydrocarbon (camphor). Furthermore, those cells which did not undergo transformation may have had or have developed resistance to the carcinogen.

The mainfestation of abnormal morphology and growth properties in the cultures treated with carcinogens indicates a change in the chemical make-up or metabolism of the cell. In this connection, spectrophotometric and chromatographic data on polysaccharides and amino acids from culture filtrates and cell lysates indicated a slight dissimilarity between the carcinogenic- and the noncarcinogenic-treated materials. It is likely that these inconsistencies reflect merely the shifting of the bacterial metabolism due to certain uncontrollable conditions such as fluctuation in incubation temperatures.

Results of experiments involving determination of methylene blue reductase activity, a qualitative and empirical method of measurement, indicate that an increased level of enzyme activity occurred in cultures grown in the presence of arenes. It is noteworthy that while the over-all reaction appeared to be nonspecific with respect to carcinogenicity or noncarcinogenicity, the quantitative differences in the reduction rate varied according to the degree of potency of the compound used.

A similar pattern of differential, quantitative enhancement was also found in lipide synthesis and lactic acid formation by the organisms. The results are similar to a phenomenon reported by Goerner (*14*). He noted an increase of total lipides in the hepatic mitochondria of rabbits injected with an arene-type of carcinogen. Similarly, Boyd and coworkers (*15*) observed a significant increase of total lipides in the malignant spleens of rats.

The demonstration of unique infrared spectra indicates a possible procedure in classifying certain atmospheric contaminants, but the data cannot be considered conclusive.

Further investigations are needed to determine the extent of the indicated implications.

CONCLUSIONS

A bioassay technique for carcinogenic activity has been developed, with bacteria as "test animals" and morphological and biochemical changes as indicators of the activity. The test material used for developmental work includes material comparable to that found in natural polluted atmospheres.

Preliminary results of the proposed technique may be summarized as follows:

1. Growth studies in shake cultures show a prolonged lag phase in a defined medium supplemented with carcinogenic arenes, whereas noncarcinogenic arenes show no lag compared to the control.
2. In complex media there is no differentiation between carcinogenic and noncarcinogenic arenes with respect to growth studies.
3. In heart infusion broth supplemented with carcinogenic arenes a large number of "giant," mottled, and reticulated cells are observed. Atypical cells are completely absent in HIB supplemented with noncarcinogenic arenes.
4. Unique bands are observed in the infrared spectra of the lipide component of organisms grown in the presence of carcinogenic arenes.
5. Quantitative differences are observed in the total lipide production and the lactic acid production of cells grown in the presence of carcinogenic arenes as compared to the control and to those grown in the presence of noncarcinogenic arenes.
6. Quantitative differences are observed in reductive reactions involving methylene blue for cells grown in the presence of carcinogenic arenes as compared to the control and to those grown in the presence of noncarcinogenic arenes.

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DISCUSSION

DR. KATZ: I would like to comment that I think this work of Dr. Won and Dr. Thomas fills an urgent need which has been evident for many years in the bioassay of polycyclic hydrocarbons. The remarkable fact about this procedure is the small quantity of material and the short length of time necessary for the test. I would like to ask one question. Have you tried other organisms in your test procedure? Have you tried chrysene or coronene or some of the other doubtful carcinogenic hydrocarbons in your enzyme test?

DR. THOMAS: There have been a number of organisms tried and comparable results have been obtained. We were looking for an organism that was very stable, that would not undergo mutation to any great extent, and *Bacillus megaterium* was the best that we could find, although we do not believe that it is the ultimate. Right now we are satisfied with it and going on with the work, but as a side line we are looking at other organisms.

We have tested a number of other compounds which have been found in polluted atmospheres and have been identified and are conventionally classed as either being carcinogenic or noncarcinogenic. Tests for carcinogenicity are usually based on whether a tumor can be induced in mice. If the mouse dies a normal death before the material has a chance to become operative, then the material is classified as noncarcinogenic. However, in our test we do not have this time factor and so it might be possible that this same material is actually weakly carcinogenic.

DR. LYONS: I want to disagree with the evaluation of this test in that too limited a number of compounds have been tested. For proper tests of this sort and for anticarcinogenic studies, we should strive to test compounds which are sterically and electronically similar and yet have widely differing physiological effects. A good range of compounds to test would be the methylated benz[*a*]anthracenes.

DR. THOMAS: Well, this probably would be incorporated into the program. We realize this particular point. Initially, we wanted to look at compounds which might be found in the polluted atmosphere to see if there was any possibility for the use of such a test. However, the amount of methylated compounds in the atmosphere is low. Our test should be related to the initial work that was done on the mouse with the same type of compound. It should be extrapolated out into the work on the bacteria. We just have not done it. We have only done preliminary work.

DR. FALK: I presume you are all aware of the fact that there is another test being developed on paramecium, and this test completely depends on light inactivating the organism and killing it. Do you eliminate light while these organisms are growing? If they are exposed to light your test might be somewhat similar to the one on paramecium.

DR. THOMAS: We are aware of this other test. Ours, of course, is with bacteria.

DR. FALK: Is yours in darkness?

DR. THOMAS: No, it is not. We do not make any effort to keep it in the dark. Our cultures are grown in the shake incubator which has a glass window on the top, but we make no effort to cut out the light.

MR. BEGEMAN: I think I must have missed a point in connection with one of the slides shown. You said about 20 minutes of exposure was required to obtain the slide. Is this a radioactive view or fluorescent?

DR. THOMAS: That was fluorescence. We were exciting the benzo[*a*]pyrene which is fluorescently visible when excited with ultraviolet light. If you look at the cells under the microscope with ultraviolet light, you cannot detect any fluorescence, but when you use the camera instead of your eye for a long period the small amount of fluorescent energy burns into the film and you get the picture that you saw. It took 20 minutes for that.

DR. VAN DUUREN: I would like to ask, Dr. Thomas, to what do you ascribe the three peaks in the infrared of the lipide in which methylcholanthrene has been used?

DR. THOMAS: Well, two of them are out in the fingerprint region, so I do not want to make any guess there. The third one, if I am not mistaken, is in the carbonyl region.

The Newt Test in Relation to Investigation on Carcinogenic and Cocarcinogenic Substances[1]

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SUMMARY

The use of rapid tests on the newt in carcinogen screening is discussed. Data are presented on the carcinogens of tobacco-smoke condensate.—NCI Monogr. No. 9: 71–73, 1962.

THE VERY long period of time necessary for tumors to appear in treated animals is still a major problem in investigations of carcinogenic and cocarcinogenic substances (air pollutants, tobacco smoke, etc.) in the environment. This is true particularly with mammifers such as mice and rats, which are considered specially capable of a response to carcinogenic substances.

A satisfactory solution has been found that considerably reduces the length of the latent period for induced tumors produced by certain carcinogenic and cocarcinogenic substances. A perfect type of batrachian, the newt, is utilized. It has long been established that the epidermis of the newt responds very rapidly—within a few days—to subcutaneous injection of extremely diluted oily solutions (*1*, *2*). Tests have shown that the reaction appears in two somewhat distinct stages: the stage of hyperplasia and the stage of infiltration of the subcutaneous tissues by epidermal cells.

From the histological point of view, various pictures can be observed; however, there are always in the injected zone two processes, hyperplasia and infiltration, which can be observed in the epithelial cells (*3*).

From the qualitative point of view, the newt test is positive only in the case of substances that are carcinogenic to mice and there is a close parallelism between this test and the test of the degeneration of sebaceous glands of the epidermis of the mouse (*3*).

It is possible to appreciate quantitatively the neoplastic power of chemical substances by noting the intensity of the reaction, in other words by the extension and the penetration of the infiltration on several animals simultaneously treated in the same way (*4*). As far as the carcinogenic aromatic hydrocarbons are concerned, we observed that they still have a positive response 18 days after injections of the following concentrations:

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[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

Dibenzanthracene	10^{-10} g per liter
Methylcholanthrene	10^{-9} g per liter
Benzopyrene	10^{-5} g per liter

The systematic utilization of the newt test in 1956 studies of the chemical composition of tobacco smoke revealed the carcinogenicity and cocarcinogenicity of the compounds given in table 1 (*5*).

TABLE 1.—Carcinogenic and cocarcinogenic activity of some hydrocarbons in the newt and mouse tests

Substance	Reaction (epidermis)		Action in mice	
	Newt	Mouse	Action	Type of tumor
Benzo[*a*]pyrene	+++	+++	Carcinogenic Cocarcinogenic	Epidermis Mammary gland
Dibenzo[*a,i*]pyrene	+++	+	Carcinogenic	Epidermis
Benz[*a*]anthracene	++	±	Carcinogenic	Epidermis
1*H*-benzo[*a*]cyclopent[*h*] anthracene	++	+	Carcinogenic	Epidermis
9*H*-benzo[*e*]cyclopent[*i*] anthracene	++	±	Carcinogenic	Epidermis
Aliphatic hydrocarbons (paraffins)	++	±	Carcinogenic Cocarcinogenic(?)	Epidermis Epidermis
Polymerized substance	++	— ++	Carcinogenic Cocarcinogenic	Epidermis Hematopoietic system Epidermis

The compounds isolated in chemical studies by Dr. J. Bonnet were tested on the newt. Substances that gave a positive response on the newt were then tested on the mouse.

Recent investigation of aliphatic hydrocarbons (paraffin) by Dr. Aubort, in our laboratory, showed that these hydrocarbons have a light but obvious carcinogenic effect on the epidermis of the mouse. The experimental conditions chosen did not allow us to establish with certainty a possible cocarcinogenic power (*6, 7*).

As for the brown-colored polymerized substances, they do not produce cancer at the injection point. At other points, however, they act as cocarcinogens on tumors produced by benzo[*a*]pyrene and on spontaneous leukemias of the mouse (*8*).

The newt test was extremely useful to us for the investigation and identification of carcinogenic and cocarcinogenic compounds contained in tobacco smoke. Its utilization should be equally useful for investigations of air pollution.

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Methods of Sampling, Separation, and Analysis of Air Pollutants: Special Requirements for Carcinogenic Bioassay [1]

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SUMMARY

The value of samples collected by present sampling methods is quite limited for experimental studies in carcinogenesis. Larger quantities of sample material are required for administration to large numbers of animals under various conditions. Only the particulate fraction of the total air pollutants is now available for bioassay; new methods are needed for the collection of the full range of pollutants. The samples probably are modified by collection, processing, and analytical methods, by the lapse of time between collection and administration, by interaction among components, by climatic conditions, and by local geographic variations. Sampling methods that eliminate or compensate for these modifications are needed so that the material adminstered to animals more nearly represents the air contaminants in the sampled area. As these new methods are developed, refined techniques for carcinogenic bioassay also must be developed.—NCI Monogr. No. 9: 75–80, 1962.

THE OBJECTIVE of this seminar is to exchange information on methods of sampling, separation, and analysis of air pollutants and to compare the data for organic substances suspected of playing a role in the carcinogenic process. Since bioassay for carcinogenicity may be considered a special kind of analysis, a review of the results of the somewhat limited investigations on the carcinogenicity of atmospheric pollutants seems appropriate. At the same time the limitations of present bioassay methods and the materials available for testing should be recognized. Finally, the special requirements for the collection, separation, and analysis imposed by such testing should be studied so that these techniques can be improved and developed and thereby the value of bioassays can be increased and the development of better ways of evaluating carcinogenicity promoted.

The study of the carcinogenicity of atmospheric pollutants by the administration of dusts from the air is not a recent innovation. In 1922 Passey (*1*) reported the production of malignant tumors in mice by the cutaneous application of an extract of household chimney soot, a major source of air pollution in England at that time. Campbell (*2*) in 1934 published the results obtained in the exposure of mice to the inhalation of dust obtained from tarred roads. Compared to the control group there was a tenfold increase in the number of primary lung tumors among the animals exposed to the dust. Cancer of the skin developed in 70 percent of the exposed mice surviving long enough. Campbell (*3*) also exposed mice by skin painting to tar obtained from road dust and chimney soot and found both contained potent carcinogens.

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[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cininnati, Ohio, August 29–31, 1961.

Siegel and Shear (*4*) in 1938 collected dust by the soot-fall method at 150 locations in New York City and administered extracts to mice by subcutaneous injection. No tumors were found at the site of injection and no noteworthy increase in number or size of pulmonary tumors was observed.

McDonald and Woodhouse (*5*) exposed mice by inhalation and skin painting to atmospheric dust from two sources, the air-purifying screens of the Birmingham General Hospital and flat-roofed buildings in central Birmingham. The incidence of lung adenomas in the mice exposed to the dust both by inhalation and painting was somewhat higher than in the control groups.

In 1942, Leiter, Shimkin, and Shear (*6*) published results of examination of 9 specimens of dust from the atmosphere for carcinogenic potency. Dusts were obtained from several cities by various methods including electrostatic precipitation, filtration through cloth, and labyrinth precipitation. Unextracted dust, as well as benzene and ethyl ether extracts, was injected subcutaneously in the right axilla of mice. No tumors were produced by the unextracted dust, but sarcomas were observed in 18 of an effective total of 291 mice that received a single injection of the tar extracted from the dust.

Because of objections to the collection methods used in the previous studies, Leiter and Shear (*7*) developed a large-capacity collector utilizing filter paper to obtain atmospheric dust samples from 5 industrial cities. After the subcutaneous injection of benzene extracts of the dust, tumors developed at the site of injection in 30 of 372 mice or 8 percent of those surviving 5 months or longer. The differences between the several cities were not conclusive.

Clemo, Miller, and Pybus (*8*) tested three chemical fractions separated from industrial smoke in an English city by skin painting of mice. Two of the fractions produced malignant tumors and papillomas.

In a study of material collected from the atmosphere of Los Angeles, Kotin, Falk, Mader, and Thomas (*9*) supplemented chemical studies with biological tests for carcinogenicity. A benzene solution of the extract from natural smog was applied to the skin of mice. At the time of appearance of the first tumor, 31 of 76 mice were surviving; 13 of these developed tumors.

In addition to the biological testing of smoke and dust from the atmosphere a number of investigators have studied these materials to identify and isolate known or suspected carcinogens, while others have investigated the carcinogenicity of combustion products, industrial wastes, and other materials that are discharged into urban atmospheres.

Utilizing materials collected, fractionated, and analyzed by Tabor and Sawicki, of the R. A. Taft Sanitary Engineering Center, Hueper, Kotin, Payne, and Falk, in 1957, began the carcinogenic bioassay of 4 organic fractions of the particulate phase samples of air pollutants collected monthly from 8 American cities. The procedures and the preliminary results were presented at the 53d Annual Meeting of the Air Pollution Control Association held in Cincinnati on May 25, 1960. The final results will be published (*10*) in the *Archives of Pathology.*

All studies mentioned demonstrate and emphasize the limitations of present carcinogenic bioassay techniques and of methods for collecting and recovering materials for such bioassays. First, only a portion of the pollutants in the air being sampled is collected. Except for whatever may be adsorbed either by the collecting medium or by the material that has been collected the gaseous phase is absent from the sample. Generally only the organic fraction, or a part of it, is recovered from the collected particulate matter. The omission of gases and constituents that are not soluble in the agent used for extraction may result in the loss of a carcinogen, or carcinogens, or a substance which plays a contributory role in the carcinogenesis process. The effect of collection and processing methods on carcinogens that may be in the air is not known fully. Prolonged sampling periods, for example, may result in the oxidation of a carcinogen to a compound that is not cancer-producing.

Another important consideration is the change that may occur between the time pollutant materials enter the air and the time they are collected. Climatic conditions may modify the composition of materials in the atmosphere either to reduce or enhance tumor-producing properties. Reference to the extensive research on the components of the Los Angeles atmosphere will give some suggestion of the potential changes that may take place after combustion products, industrial wastes, or other pollutants enter the air. Similar or even more drastic chemical changes may be brought about by the interaction of compounds originating from different sources resulting in an increase in carcinogenicity. Conversely, interaction may result in reducing the carcinogenicity of samples administered to animals. As an illustration, Payne (*11*) found that increasing amounts of chromates administered with a constant dose of benzo[*a*]pyrene resulted in a decreasing number of tumors in mice. Chemicals, metals for example, that may be in the air in very low concentrations may serve as catalysts and produce reactions that would not occur otherwise. On the other hand, transient compounds that may be in a plant effluent may no longer be present when the material is analyzed or administered to animals.

The number and location of air-sampling stations within a given area in relation to sources of pollution, prevailing meteorology, topography, and population distribution are of utmost importance in any study of the relation of air pollution to health effects. In the studies described it must be recognized that the materials tested in animals may not be representative of the material actually in the air from which the sample was taken; if the sources of pollution are numerous in the area, the air from which the sample is withdrawn may not contain the contaminants in the proportion that they exist in the area as a whole. If there are not enough sampling stations, or if they are located without regard to prevailing wind direction, a carcinogen arising from a limited geographical area may be omitted from the test material. In the comparison of the results of either chemical analysis or bioassays with observed cancer rates in the population of the city under study, correlation should not be expected if the population distribution in the city is such that large segments are not exposed to the air from which samples are taken. These considerations would indicate that investigations should be planned in which smaller geographical areas are studied more intensively.

In summary, to obtain better results from bioassays a number of aspects of sampling, as well as bioassay methods themselves, need to be developed and improved. All substances comprising the air-pollutant material should be collected and recovered in a form that can be administered readily to animals, preferably without the use of a vehicle. The material should include not only organics but minerals and the gaseous phase. The sampling and collecting apparatus should be capable of collecting the material in a short time and in a manner that will minimize changes in the state of the substances and interaction between components. Taking into consideration meteorology, topography, and sources, sampling stations should be located in sufficient number and operated in a manner to insure that the material administered to animals is representative of the air contaminants in the area under study. Sufficient material should be recovered by sampling so that it can be administered to a large number of animals over a long period; preferably more than one species of animal and more than one route of administration should be used. As methods for sampling, separation, and analysis are improved, techniques for carcinogenic bioassay must be modified and refined and new approaches developed.

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DISCUSSION

DR. HOFFMAN: For the record, other short-term tests have been described before by Suntzeff and coworkers (1955) using the sebaceous glands of mouse skin as a screening technique for the carcinogenicity of chemicals as well as combustion materials. These authors pointed out, however, that their short-term tests cannot substitute for long-term studies.

DR. KATZ: I think Dr. Payne has discussed several points of great importance to workers in this field. Anthracene, naphthalene, and phenanthrene are not usually collected on a filter because of their high volatility. We need a more versatile method of collection.

DR. VAN DUUREN: Dr. Payne, in one of the tables shown you listed an oxygenated neutral fraction and its carcinogenicity. Could you define these materials chemically?

DR. PAYNE: Since that was part of the Southern California work, I think Dr. Falk could answer that question.

DR. FALK: The material was obtained from the Robert A. Taft Sanitary Engineering Center. It is a brown oily material and is obtained from the benzene-soluble fraction of airborne particulates. The benzene-soluble fraction is usually chromatographed on alumina. The aliphatic fraction is eluted first, followed by the aromatic and oxygenated neutral fractions, in that order. The phenols and acids are retained on the column.

MR. TABOR: I think Dr. Falk pretty well covered this situation, although I might briefly outline the procedure we use. We first removed the acid material and the basic material. The third fraction, the neutrals, was then chromatographed. The aliphatic hydrocarbons were eluted first, then the aromatic hydrocarbons, and finally the oxygenated neutrals. They were neutral chemically, and they certainly did contain oxygen.

DR. VAN DUUREN: My interest in this is because of the reported carcinogenicity of epoxides, hydroperoxides, and related materials. I was wondering whether any of these types of compounds would be in that particular fraction.

DR. HOFFMANN: May I ask a question? Dr. Van Duuren, would you not expect to find dibenzacarbazoles, which you so well identified in cigarette smoke tar, in this oxygenated fraction?

DR. VAN DUUREN: Yes, they would be in the neutral fraction.

DR. KATZ: I would like to ask a question about the Los Angeles results. One would expect that with the 3 million or more motor vehicles operating in the Los Angeles area and the evidence for the presence of carcinogenic hydrocarbons in automobile exhaust gas, a greater potential carcinogenicity for the Los Angeles atmosphere exists than is shown in the data reported by Hueper and his coworkers. Yet the Los Angeles samples were virtually the least carcinogenic. Is there any explanation for this?

DR. FALK: The problem in Los Angeles is that the quantity of benzopyrene in the atmosphere is very small, probably because we do not use coal. There is no benzopyrene to speak of compared to Chicago, New York, and other coal-burning cities, although there is plenty of benzopyrene in the automobile exhaust. However, we found another carcinogenic agent, epoxide, but the probability of forming epoxides in automobile exhaust is actually quantitative. The low incidence of tumors in the Los Angeles area in this mouse study probably will be contradicted by the actual physiological data obtained in Los Angeles about 1975. Actually there are many other factors involved in carcinogenesis.

DR. BADGER: If I may return to Dr. Van Duuren's question about the epoxides. Would it not be true that the epoxides, if present, would be found in the aliphatic hydrocarbon fraction? I would also like to ask whether there was any oxygen in this fraction.

MR. TABOR: We did not analyze any samples to see whether oxygen was present. Most of our measurements were limited to infrared examination. The aliphatic extracts that we did send out for experimental purposes showed remarkably true pictures of aliphatic hydrocarbons. We did not find any indication of anything other than the pure hydrocarbon bands; however, this does not rule out the possibility of a slight amount of oxygen being present.

CHAIRMAN SAWICKI: I would like to make one comment here. Even if epoxides were present, the infrared spectrum would not show them, especially if they were present in small amounts. However, we have developed some new colorimetric methods for alkylating agents and epoxides. With this test we have obtained positive results with some of the aliphatic fractions. This seems to indicate that an alkylating agent, possibly an epoxide, is in some of these fractions.

DR. ALTSHULLER: I have a comment on these epoxides. I do not think we should forget, as Dr. Payne has pointed out, that there are many other organic compounds present beside the organic particulate fraction. There is an excellent probability that many of the lower molecular weight epoxides do not appear in this organic particulate fraction, but in the gas phase. As a matter of fact, in work on the vapor-phase photooxidation of olefin and NO_2 mixtures, which was done at Stanford Research Institute, epoxides were detected and determined in simple systems by means of gas chromatography in the C_4 and C_3 range. I am sure that the C_5 and C_6 epoxides are also in these mixtures. So whether or not they appear in the particulate fraction, I think it is likely that they appear in the gaseous fraction.

DR. LYONS: As a possible extension of the intratracheal method of bioassay, have you ever considered employing the method of Horning; it seems to have been neglected. He dissected out the trachial bronchial tree of young mice, segmented the trachea and major bronchi, and added carcinogens in crystallized form to these segmented parts that were then implanted into adult hosts of the same strain. Many such implants produced carcinomas.

DR. PAYNE: We have not tried anything of that sort. We have followed pretty much the routine methods of painting, subcutaneous injection, and injection of various sites in the animal. We are planning in the near future to expand the development phase of carcinogenic screening. Dr. Weisburger, who will be coming with us, will be looking for different methods to try, and I am sure he will be interested in that method as well as others.

An Assessment of Factors Concerned With the Carcinogenic Properties of Air Pollutants [1]

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SUMMARY

The presentation is devoted primarily to the representative carcinogenic polycyclic hydrocarbon, benzo[*a*]pyrene. The biological significance of particle size of naturally occurring benzo[*a*] pyrene is considered. Along with carcinogenic polynuclear hydrocarbons, carcinogenic agents of a different chemical nature occur in the atmosphere. These may act as cocarcinogens or may otherwise affect the action of benzo[*a*]-pyrene. Noncarcinogenic chemical and biological agents in polluted air also may affect the biological action of carcinogenic agents, as may the simultaneous presence of related compounds. Anticarcinogenic action may be related to the ratio of the compounds to one another and to the stability of both carcinogenic and noncarcinogenic compounds in the atmosphere. In vehicle exhaust studies further modifying factors are the conditions of engine operation and the variations in proportion of compounds due to atmospheric exposure. Further, the solvent or vehicle effect modifies the rate of entry of carcinogenic agents through epithelial surfaces and their subsequent anatomic distribution. The metabolism of polycyclic aromatic hydrocarbons in the liver and associated metabolic alterations are considered.—NCI Monogr. No. 9: 81–89, 1962.

IN ADDITION to the quantitation of known and suspected carcinogenic compounds identified in polluted air, several factors have been established as significant in the evaluation of the carcinogenic properties of polluted air. These ancillary factors may contribute in part to the varied epidemiologic pattern of lung cancer in relation to polluted air and other environmental agents.

The factors considered will be discussed under the headings: physical, chemical, physiological, and pathological. As will become apparent, the extent of knowledge in relation to each varies, and considerably more work is necessary for the complete elucidation of the role of polluted air in the pathogenesis of cancer.

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[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

PHYSICAL FACTORS

In the evaluation of polluted air as a carcinogenic hazard, the significance of particle size is critical. The feasibility of respiratory tract entry, the level of deposition and retention of particles in arborizations of the tracheobronchial tree, and the elution of carcinogenic hydrocarbons from soot particles, after ingestion by pulmonary macrophages, are all in large measure reflections of particle size. Particles so large as to be incapable of either host entry or penetration beyond the upper respiratory tract are of little concern, whereas those lying in the 100 mμ to 2μ size range are indeed of greatest biological significance. The latter readily penetrate and are retained in the tracheobronchial tree, and in the presence of cytoplasmic or tissue proteins the adsorbed carcinogenic materials are easily eluted. Particles smaller than 100 mμ are still retained in the tracheobronchial tree; however, elution of hydrocarbons from these small particles does not occur. Particles below 40 mμ in diameter are of little biological interest, since they primarily remain suspended in tidal air.

The significance of particle size in relation to carcinogenicity is further emphasized when the effect of solvent action and elution within the host is compared with the effect of elution under laboratory conditions. The smallest particle size from which elution of carcinogenic aromatic polycyclic hydrocarbons (APH) from soot can be demonstrated under physiological conditions is 100 mμ. In contrast, with laboratory solvents such as benzene, elution at a 100 percent level of efficiency is possible with particles down to 80 mμ. Less efficient elution can be demonstrated in particles down to 40 mμ, whereas below 30 mμ solvent action for elution of APH cannot be demonstrated under any circumstances. It is in this size range that particles will actually adsorb or remove APH from solution. In addition to the more generally recognized sources of soot, a ubiquitous air pollutant, it is of interest to mention rubber tire dust, which, despite the modification of the soot by embedding in rubber, still permits the rapid elution of APH by physiological or *in vivo* media.

The rate as well as the quantitative aspects of elution of APH from soot is an additional consideration of major importance. The smaller APH are uniformly more easily eluted than the larger ones, and this observation is consistent, independent of whether laboratory solvents or protein solutions are used. As a result, there is a nonuniform release of APH from soot particles after exposure to physiological solvents. This varied release of APH modifies the interaction of one with another. As will be discussed, the inconstant pattern of release has certain implications in relation to anticarcinogenesis.

The final physical factor to be considered is that concerned with the stability of APH on soot incidental to atmospheric exposure. In polluted air a number of APH disappear quite rapidly from soot mainly by sublimation and to a lesser degree by oxidative destruction. The rate of disappearance of APH from soot is maximal in the light, whereas little destruction can be noted in the dark. In general, small APH disappear most rapidly, whereas some of the larger ones persist unchanged. These same larger APH are not eluted to any extent by solvents.

CHEMICAL FACTORS

Independent of source, incomplete combustion of carbonaceous matter has yielded an essentially similar spectrum of APH on soot. In relation to the operation of gasoline engines, it is possible to alter this spectrum slightly by altering the conditions of engine operation. These changes are minimal, however, and are primarily manifested in an alteration in the proportion of the various APH rather than the appearance or disappearance of particular ones. The smaller APH appear to be consistently produced in higher concentration than the larger ones, and, as will be noted, it is of particular significance that the simpler APH, like anthracene, phenanthrene, pyrene, etc., can be detected in 10 times the concentration of benzo[*a*]pyrene. APH closely related to benzo[*a*]pyrene reduced the carcinogenic potency of this carcinogen when it and a related compound were dissolved in a lipide solvent and injected subcutaneously into mice. A 50-percent reduction in tumor yield was observed in the mice receiving both benzo[*a*]pyrene and related APH.

The phenomenon of competitive inhibition has been demonstrated for a broad group of related compounds, and in a more limited way studies have been performed on potential inhibitors present in polluted air. It has been further established that certain very closely related, partially reduced hydrocarbons could completely inhibit tumor production by benzo[*a*]pyrene. This is in contrast to the partial inhibition by simpler polycyclic hydrocarbons. In this group phenanthrene, anthracene, and pyrene demonstrate the same relatively low level of inhibition. The higher APH show little inhibitory effect on benzo[*a*]pyrene, presumably as a result of the ratio to the latter, in which they occur. They occur in a ratio of 1:1 or less in soot, whereas the small APH are usually present in a ratio of approximately 10:1.

An additional factor, particularly in relation to differences in the rate of elution of APH from soot, is the effect of variations in time between the administration of the inhibitor and the application of the carcinogen. Variations in time are reflected by differences in the pattern of inhibition. Thus, inhibition can be achieved to the same extent when two compounds are applied simultaneously or when the inhibitor is applied for periods varying from 7 days before carcinogen administration or 7 days after. As time intervals are increased beyond these points, however, inhibition is reduced and ultimately disappears. The solvent system used in inhibition studies appears to have a modifying effect on the relationship of the inhibitor to the carcinogen. Data to explain the mechanism of solvent effect are as yet unavailable.

The survival time of soot in the air must also be considered in terms of any carcinogenic effect it may possess. As noted, the smaller APH disappear from soot in polluted air at a more rapid rate than the larger ones. Thus, a fresh sample of vehicular exhaust or soot collected at any source may be less carcinogenic than soot from a similar source collected from polluted air at a later date. Within the host the rate at which APH are eluted from soot by proteins is of lesser significance in this connection when contrasted with the situation in polluted air. In the host the time interval for biological effect is sufficiently short so that complete elution of active anticarcinogens and carcinogenic agents can occur within the critical period of 14 days. The significant effect of the modifying medium, *i.e.*, whether lipide or aqueous, has been observed in experimental studies.

Studies have been performed on factors concerned with the inhalation and retention of air pollutants in the tracheobronchial tree. In addition, their penetration through the mucous barrier overlying the respiratory epithelium into the underlying cells and their subsequent systemic distribution and metabolism have been investigated. Factors that interfere with normal physiological activity of the respiratory tract have been identified and their mode of action has been elucidated.

PHYSIOLOGICAL FACTORS

The physiological activity of the respiratory epithelium is adversely affected by chemical agents in polluted urban atmosphere. This is manifested by a slowing of the flow of the mucous stream, alteration in the physical properties of the mucus, and changes in ciliary activity. The response may be quantitated by measuring the flow rate of particles on isolated strips of ciliated mucus-secreting epithelium. Although quantitative differences exist in different species of animals, the qualitative pattern is a relatively constant one. The impingement of chemical agents produces a more intense and longer-lasting effect than does the passive flow of agents over the epithelium. The demonstration *in vitro* of slowing of mucous flow can be paralleled by the use of studies *in vivo* in which abnormal retention of soot can be demonstrated after exposure of intact animals to chemical agents in polluted urban atmosphere. The changes in mucous flow observed during quantitative studies are associated with histologically demonstrable changes in the epithelium. Interference with mucous flow facilitates the accumulation and retention of carcinogenic particulates so that a biologic environment for their optimum action is created.

Ciliary activity and mucous secretion appear to be locally controlled by acetylcholine activity. Pretreatment of the eqithelium with parasympatheticomimetic agents provides a buffering or protection against the effects of irritant aerosols so that physiological or occasionally enhanced rates of mucous-stream flow result after their application. Agents studied include: 1) acetylcholine and choline-like drugs, 2) cholinesterase inhibitors, and 3) derivatives of nicotinic acid with parasympatheticomimetic properties. The protective effect can be demonstrated both *in vitro* and *in vivo*, and it persists for periods up to 6 hours. Concentrations well below those of local or systemic toxicity produce the desired response.

A different approach to the problem of host defense in air pollutants was taken by a study of the efficiency of the liver in detoxification of benzo[*a*]pyrene and other introduced APH. It was found by the use of radioactive benzo[*a*]pyrene that the penetration of various sites occurs very rapidly. Penetration of the skin occurs in as little as 6 hours, and in the mouse radioactivity disappears from the epilated skin in 30 hours. These experiments were confirmed on the epilated skin of rats, and the appearance of the radioactivity in the bile was followed for periods up to 30 hours. After a slow start increasing amounts of radioactivity were excreted. It was noted here that the solvent has little effect, *i.e.*, identical results were obtainable whether the benzo[*a*]pyrene was applied in benzene, in a lanolin base, or in other vehicles. It was also of interest to note that as the dose per unit area was increased, so was the amount excreted in the bile.

Of particular interest in studies of penetration of tissues were those related to the lungs and the gastrointestinal tract, inasmuch as most of the agents in polluted air enter the host by these routes. The most rapid penetration was observed in the lungs after the infusion of benzo[*a*]pyrene into the trachea. Virtually no absorption occurs from the stomach, whereas considerable absorption again occurs from the small and large intestine. The solvent effect here proved a significant one, resulting in benzo[*a*]pyrene activity after subcutaneous injection into mice. No tumors were produced when a protein solution or an aqueous suspension of benzo[*a*]pyrene was used. In short-term studies it was noted that when benzo[*a*]pyrene was administered in protein or aqueous suspension, it was readily eliminated in the feces within a few days. In contrast, tumors were produced, and elimination of benzo[*a*]pyrene in the feces was markedly reduced when a lipide medium was chosen for injection.

Thus, the effect of the vehicle on the availability for detoxification and consequently on carcinogenic action is marked. The rate of entry of benzo[*a*]pyrene into the circulation via the tracheobronchial tree appears independent of the medium, *i.e.*, whether lipide or aqueous. In earlier studies we have shown that after the intratracheal administration of benzo[*a*]pyrene in either triethylene glycol or saline, metabolites in the bile can be readily detected, but in relatively low concentration. The addition of a dilute protein solution to the aqueous benzo[*a*]pyrene markedly increases the rate of absorption and biliary excretion after intratracheal administration. The barrier effect of the mucus on the respiratory epithelium is apparently neutralized by the abnormal presence of protein. In contrast, the addition of mucus to aqueous benzo[*a*]pyrene markedly reduces the absorption of benzo[*a*]pyrene to a level well below that of the controls. This inhibitory effect of the added mucus persists even when protein solution is added to the mucus-aqueous benzo[*a*]pyrene complex. Under physiological conditions the absorption of benzo[*a*]pyrene through the respiratory epithelium is to a remarkable degree governed by the relative concentrations of protein and mucus as well as the physical state of the benzo[*a*]pyrene.

After entry into the circulatory system benzo[*a*]pyrene is rapidly eliminated by the liver, and metabolites are detected in the bile within a few minutes. During this time the benzo[*a*]pyrene is oxidized rapidly in the 6 position and conjugated to the glucuronide. Further oxidation takes place to the 1,6- and 3,6-dihydroxybenzo[*a*]pyrene and to a small extent to the 6,12-dihydroxybenzo[*a*]pyrene. All compounds are excreted in the bile as conjugates with glucuronic acid and to a smaller extent as sulfates. About 30 minutes after the introduction of benzo[*a*]pyrene an additional product is formed, which ultimately becomes the preponderant one. This metabolite is 3-hydroxybenzo[*a*]pyrene; it is excreted in conjugation with glucuronic acid and also as a glucuronide and sulfate after reduction to its dihydro-dihydroxy derivatives. In addition to these identical metabolites, other investigators, as well as ourselves, have detected, but have not characterized, several others. Of interest in this group is the class of X_1 metabolites, one of which on mild acid treatment reverts to benzo[*a*]pyrene. This compound accounts for only 3 to 5 percent of the total metabolites, but it is of considerable interest inasmuch as it may revert to benzo[*a*]pyrene after this carcinogen has supposedly been metabolized and detoxified. Further studies on the *in vivo* conditions for its reactivation are under way. It appears that virtually all other metabolites of benzo[*a*]pyrene possess either extremely low or no carcinogenic activity at all.

PATHOLOGICAL FACTORS

Inasmuch as the liver is the primary site of metabolism of APH, injury to this organ results in interference with the normal detoxification of carcinogenic APH. In consequence we have studied the effects of injury to the liver induced by injection of CCl_4 in mineral oil. A dose of 0.06 ml per 100 g wet weight of rat was sufficient to cause liver damage that was apparent 6 hours after injection and persisted for 4 to 5 days. Experiments were performed to obtain information on the ability of the liver to re-establish normal metabolism after injury. One method of study used was the measurement of the specific activity in the bile after administration of radioactive benzo[*a*]pyrene. It was noted that the physiological pattern of bile excretion and the appearance of benzo[*a*]pyrene metabolite were considerably altered. Instead of the highest specific activity appearing at 30 minutes after injection, the peak was not reached until 3 hours after injection. The magnitude of activity at that time was only 30 percent of the normal. The retained isotopically labeled benzo[*a*]pyrene was not found in excessive amounts in the organs of excretion. The liver and kidney contained only normal amounts, and large quantities of radioactivity apparently remained at sites as yet unidentified.

The types of metabolites were normal except for that group of metabolites formed from 3-hydroxybenzo[*a*]pyrene. Some of the compounds were present in smaller amounts and showed less conjugation than those seen normally. Physiological activity of the liver is probably a factor of considerable importance in the evaluation of the activity of environmental carcinogenic agents. Experimentally, limited injury to the liver from which recovery ensues within 1 week appears to cause the retention of large amounts of benzo[*a*]pyrene. The significance of the altered spectrum of metabolites is not known at this time.

If I may turn away for the moment from the significance of benzo[*a*]pyrene, *i.e.*, of soot in the etiology of lung cancer, I would like to mention experiments carried out on the synergistic effect of exposure to influenza virus infection and smog. The exposure of C57BL mice, a strain highly resistant to the development of lung cancer, to 3 different types of influenza viruses and artificial smog made from ozone and gasoline leads to enhanced squamous metaplasia formation and the appearance of squamous lung cancers. Mice exposed exclusively to influenza virus infection, smog, or washed air showed no similar changes. The squamous cancers produced are histologically indistinguishable from human bronchiogenic carcinoma and appear to be the first produced in C57BL mice by use of air pollution as one of the test agents. The data indicate the need for further studies on the synergistic effects of soot, smog, virus infections, and liver damage as factors in the production of lung cancer.

In summary, it must be stated that the complexity of the etiology of lung cancer becomes more staggering as new factors are elucidated and more is understood about the many facets of the physical, chemical, physiological, and pathological factors concerned with the production of cancer.

I have specifically refrained from commenting on the less-important carcinogenic agents we have detected in soot. Included in this group would be benzo[*b*]fluoranthene. In a manner similar to that of benzo[*a*]pyrene, it presumably has its inhibitors in fluoranthene and possibly benzo[*k*]fluoranthene.

Analytical methods for quantitation of polycyclic aromatic hydrocarbons in polluted air have been studied with the hope of improving existing methods. Chromatographic separation techniques have not been changed. The first step was chromatography, using neutral aluminum oxide column deactivated partially by the addition of 2 percent by weight of water. This was followed by one or another of the methods of paper chromatography described in the literature. Strips were scanned on our fluorescence scanner and spectra were obtained.

It is at this step that we sought to further improve analytical methods. The most successful and most readily available method made use of the shift in spectrum of compounds going from solution to a microcrystalline suspension. Similar fluorescent spectra like those of benzo[*a*]pyrene and benzo[*k*]fluoranthene could thus be more readily distinguished. The benzo[*a*]pyrene fluorescence completely disappears from the 400 to 500 mμ range, whereas that of the other remains essentially unchanged in a solution of aqueous acetic acid.

Additional laboratory methods included attempts to utilize the change in fluorescence incidental to change in temperature. Room temperature and liquid-nitrogen temperature were used as the two extremes. Most compounds showed improved spectra with higher intensity at low temperature. This method was used for confirmation under certain conditions. An Aminco-Kiers spectrophosphorimeter was used for this purpose. Another method attempted was phosphorescence spectroscopy. Though we have been using it for some time, it is both laborious and difficult. A combination of all available methods of fluorescence and phosphorescence spectroscopy was deemed necessary for good characterization of compounds, inasmuch as ultraviolet absorption spectroscopy could not be used most of the time because of the microquantities of material available. When feasible, spectra were obtained on a DK-2 using a "micro" cell containing 0.5 ml solution. Completely characteristic fluorescence spectra could not be obtained routinely by the use of all the instruments available to us.

DISCUSSION

DR. HOFFMANN: I have three questions. First, you showed your study of the inhibition by polynuclear aromatic hydrocarbons of the carcinogenic reactions of benzo[*a*]pyrene. On how many mice was this experiment done, and how great do you estimate the experimental deviation?

DR. FALK: The size of these groups was only 30 animals each, and the total yield of tumors in the control group was at the 50 percent level. Thus approximately 15 developed in the controls, and after the inhibition was manifested, about half that number developed. The reason that we did not subject our data to a statistical analysis or concern ourselves with distribution curves was that we had performed a large series of experiments at the same time and in all studies the findings were essentially similar. In all, we had about 20 different groups going at the same time. For our purposes one could combine quite a number of them, because the only variation between them were such factors as the dose, the time of injection, or some other variable like the solvent. We feel, therefore, that any inhibition effects we report are definite.

Various solvents for the hydrocarbons to be tested in the inhibition study were investigated. One of the worst was triethylene glycol. It is a good solvent for benzo[*a*]pyrene and it is very water soluble. As soon as it gets into the animal, it has a tendency to disappear and is dissipated all through the tissue. The benzo[*a*]pyrene settles out as a very fine precipitate. Under these conditions the protective effect and the inhibitory effect of other chemicals were absent. This effect shows that the solvent is exceedingly important, and inhibitory studies must be carried out with a variety of solvents. The solvent's properties have to be consistent with those present in the lungs.

DR. HOFFMANN: The second question concerns the application of benzo[*a*]pyrene to the skin of rats. I believe nobody has succeeded in obtaining cancer of the skin when this chemical is painted on the skin of rats, my question is why did you use rats? Was there a special reason?

DR. FALK: Benzo[*a*]pyrene was applied to the skin of rats as it was applied to every other area that we thought of significance in metabolism. We also did the same work on mice. However, in the rat we have one tremendous advantage, in addition to the fact that the animal is larger and is more easily operated on, in that the rat does not possess a gallbladder. With other species of animals available to us our experiments were ruined because of the action in the gallbladder of β-glucuronidase which is present in high concentration. All the metabolites except the sulfate are hydrolyzed to phenols and hydrophenols. When we attempted to isolate these compounds, they were so readily oxidized by the air that they disappeared before we could find them. We proved that by using radioactive tracers, and we actually found that the rate at which these phenolic compounds are destroyed is phenomenal. After we established that, we stopped using the mouse in this type of metabolic study.

DR. LYONS: To elucidate a point for Dr. Hoffmann, Schoental and Hays in Glasgow produced, I think, squamous carcinoma on the skin of rats by repeated application of carcinogens over a long period.

DR. THOMAS: This has to do with the final 3 minutes of your comment on temperature and fluorescence in that finer structure and greater intensities are obtained in the fluorescence spectra of a compound at liquid-nitrogen temperatures. Beckman is coming out with an instrument in the next 3 or 4 months that utilizes the conventional light housing. It is an attachment whereby you do not have to go down to liquid-nitrogen temperatures. It uses the water-cooled tubing and just by dropping 10 to 20° C you get increased sensitivity and fine structure. I was wondering whether you have ever done any work with temperatures outside of the extreme that you took with liquid nitrogen.

DR. FALK: I never did, no.

DR. HOFFMANN: There was work in Leningrad by the Russian group. Dikun has studied the fluorescent spectra of benzo[*a*]pyrene at different temperatures.

DR. FALK: I might mention that I have seen a fluorescence spectrum of benzo[*a*]-pyrene by a French worker who has shown the existence of about 25 different peaks. I presume none of our existing machines are capable of giving that resolution. If such an instrument were available, it would solve our identification problems. Absorption spectroscopy would not be necessary then. I do not know if this instrument is available. Does anybody know how this information was obtained?

MR. HOWERTON: It is usually taken with a very high-resolution spectrometer like a German three-prism spectrometer, and usually at a very low temperature to resolve the many peaks. There is a recent report in *Optics and Spectroscopy* of obtaining fluorescence, phosphorescence, and absorption spectra with this high-resolution prism spectrometer. The July or August issue of *Optics and Spectroscopy* even shows the spectra of many polynuclear hydrocarbons.

Analytical and Biological Studies on Gasoline Engine Exhaust [1]

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SUMMARY

Exhaust fumes of a V-8 engine were analyzed for polynuclear aromatic hydrocarbons and for phenols. Results, analytical techniques, and the method that led to the isolation of various polynuclear aromatic hydrocarbons in crystalline form are described. These results are discussed in relation to the tumor potency of exhaust fume condensate to mouse epidermis and are compared to cigarette-smoke condensate. The formation of some polynuclear aromatic hydrocarbons is related to the type of fuel and the mileage of the engine. Methods for the determination of benzo[*a*]pyrene and benz[*a*]anthracene in air pollutants are compared.—NCI Monogr. No. 9: 91–116, 1962.

AS STUDENTS of environmental carcinogenesis we have, first, to evaluate available human data linking environmental factors to a given type of cancer before interpreting laboratory studies.

What is the existing evidence on the relationship of general air pollution and lung cancer? Hammond and Horn (*1*) in their 44 months' follow-up study of 187,783 men showed that a somewhat higher lung cancer ratio exists in cities than in rural areas (text-fig. 1). This result, according to the authors, cannot be explained solely by the occupational cancer predominantly found in cities. Their study suggests, but does not prove, that general air pollution plays a role in the development of lung cancer. The urban/rural ratio of lung cancer is not of the same magnitude as the smoker/nonsmoker ratio, according to a recent paper by Wynder and Hammond (*2*) (text-fig. 2).

The fact remains, according to all investigators, that a somewhat higher rate for lung cancer exists in city areas than in rural. These data led us to laboratory studies, which are not intended, however, to

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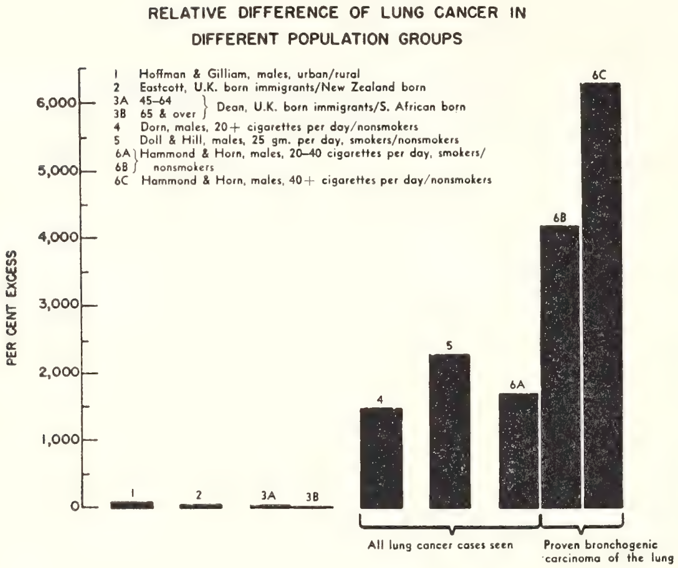
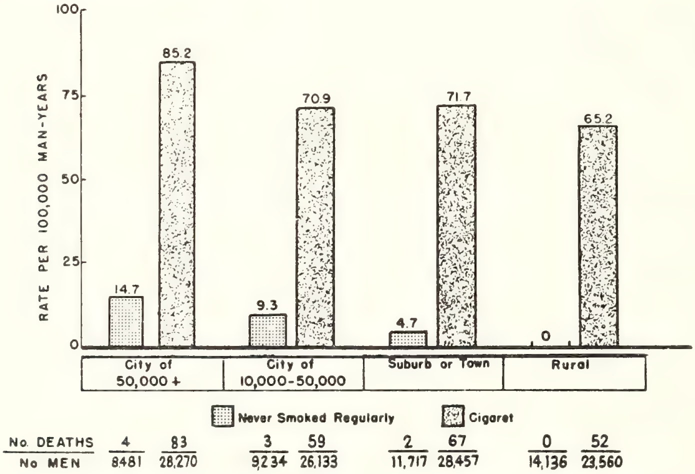
[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

[2] We wish to express our great appreciation to the Research Staff of the General Motors Corporation who made this study possible; we are especially indebted to Charles Begeman for his close cooperation. Also, we wish to acknowledge the excellent assistance of Mrs. I. Lemm-Hoffmann, Miss M. Christian, Mr. E. Theisz, and Mr. G. Rathkamp.



TEXT-FIGURE 1.—Carcinoma of lung, well-established diagnosis (excluding adenocarcinoma).



TEXT-FIGURE 2.—Relative difference of lung cancer in different population groups.

establish proof of the carcinogenicity of air pollutants to men. Human data must be relied on for such proof. But we would like to understand and be able to explain the factors involved in air pollution carcinogenesis. This explanation must correlate with the human findings.

One approach to this problem is through the close cooperation of biologists and chemists; therefore, we would like to present our chemical, analytical, and biological data.

The experimental data on the collection system for polynuclear aromatic hydrocarbons and phenols from a V-8 engine were presented earlier in this symposium by Charles Begeman, from the General Motors Research Laboratories (*3*), so that I may continue at this point with the report on our analytical data.

The dry benzene extract of the engine exhaust condensate and the benzene : methanol extract of the filter system were combined according to the ratio found and a sample of this mixture was chemically analyzed. The main portion was used as test material for carcinogenicity studies on mouse skin.

The first step in the analysis for polynuclear aromatic hydrocarbons was a partition between methanol:water (4:1) and cyclohexane. Thereby we enriched the aromatic hydrocarbons in the cyclohexane phase under separation from the hydrophilic compounds (*4*). As shown by the partition coefficients, it is possible to retain more than 99 percent of the polynuclear aromatic hydrocarbons in the cyclohexane phase after only 3 extractions (table 1).

TABLE 1.—Partition coefficients of polycyclic hydrocarbons (20° C)

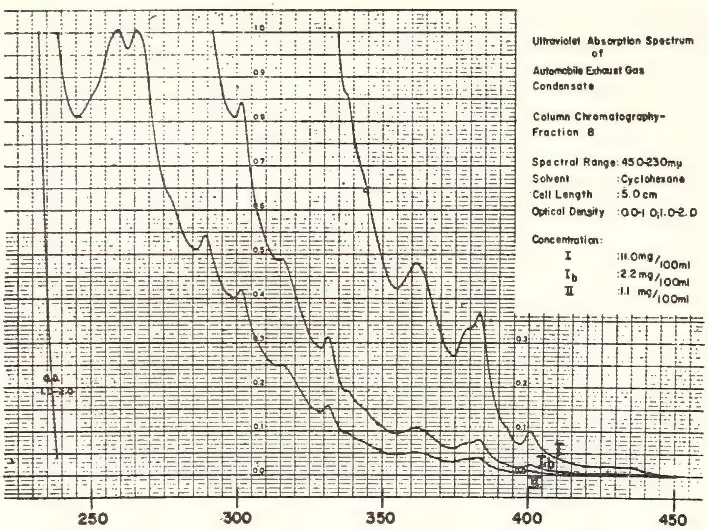
Hydrocarbon	C_c/C_m *	C_n/C_c *
Pyrene	150. 0	4. 40
Benzo[*a*]pyrene	14. 9	1. 53
Benzo[*e*]pyrene	14. 5	1. 68
Fluoranthene	140. 0	1. 80
Benzo[*b*]fluoranthene	14. 7	1. 94
Benzo[*j*]fluoranthene	14. 8	1. 75
Benzo[*k*]fluoranthene	14. 9	1. 76
Benz[*a*]anthracene	18. 6	1. 75
Dibenz[*a, h*]anthracene	14. 9	1. 80
Perylene	14. 9	1. 69
Benzo[*ghi*]perylene	14. 6	1. 77
Chrysene	14. 6	1. 65

*C_c = concentration in cyclohexane; C_m = concentration in methanol-water 4: 1; C_n = concentration in nitromethane. Coefficients are obtained after spectroscopic determination of concentrations.

In order to lose most of the paraffins which would later influence the column chromatography, we enriched the hydrocarbons further by distribution between cyclohexane and nitromethane (*4*).

The dry residue of the nitromethane extracts was chromatographed on neutral alumina (Woelm) of activity grade I. Hexane, hexane : benzene mixtures, and benzene elute the fractions which are taken according to their fluorescence observed on the column with an ultraviolet lamp of 365 mμ wavelength. The ultraviolet absorption spectrum of the main fraction of benzo[*a*]pyrene observed is shown in text-figure 3.

Despite repeated column chromatography on silica gel and alumina, we did not succeed in obtaining fractions which show ultraviolet absorption and fluorescence spectra identical with those of authentic reference compounds. For this reason we chose paper chromatography on acetylated paper as the analytical method for isolation of pure polynuclear aromatic hydrocarbons (*5*). For example, 2 paper chromatograms were sufficient to obtain benzo[*a*]pyrene identical with its reference in R_F



TEXT-FIGURE 3.—Ultraviolet absorption spectrum of automobile-exhaust gas condensate. Column chromatographic fraction 8.

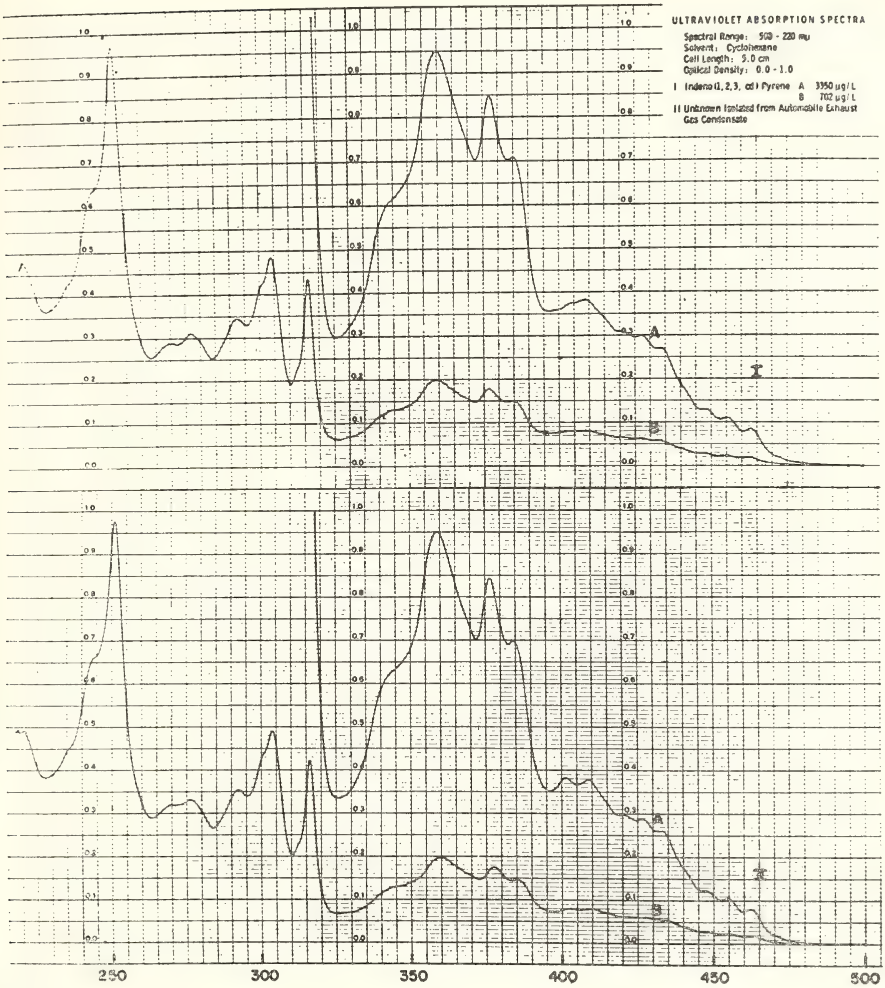
value and in ultraviolet absorption and fluorescence spectra. Other equally pure hydrocarbons can be obtained after a single paper chromatogram of the main column fraction, *i.e.*, pyrene; however, some require up to 5 paper chromatograms before they definitely show an ultraviolet absorption and fluorescence spectrum entirely identical with a reference. The latter was the case for dibenzo[*a*,*l*]pyrene.

By repeated paper chromatography we also obtained in one of the late column fractions a reddish-yellow fluorescent band, the extract of which gave a distinct ultraviolet absorption spectrum (text-fig. 4), and also a distinct fluorescence spectrum.

In checking the Wyandotte punched cards for the ultraviolet absorption spectra of the ASTM, we could not identify the spectrum of the isolated compound with any of their spectra recorded up to 1957 (*6*). In 1958 Aitken and Reid synthesized indeno[1,2,3-*cd*]pyrene and published its ultraviolet spectrum (*7*). We found this identical with our isolated compound (text-fig. 4); the fluorescence spectra were also identical.

We consider a polynuclear aromatic hydrocarbon is present in automobile exhaust gas only when we can establish identity with a reference in R_F value, ultraviolet absorption, and fluorescence spectrum or when an otherwise unknown compound does not change its spectra and R_F value after repeated paper chromatography (text-fig. 5).

When larger quantities of polynuclear hydrocarbons were determined in a sample, we were able to crystallize the hydrocarbon from the paper chromatography end fraction after vacuum sublimation. In addition, the melting point, mixture melting point, and fluorescence of the crystal served as further criteria for identity with a known hydrocarbon (table 2).



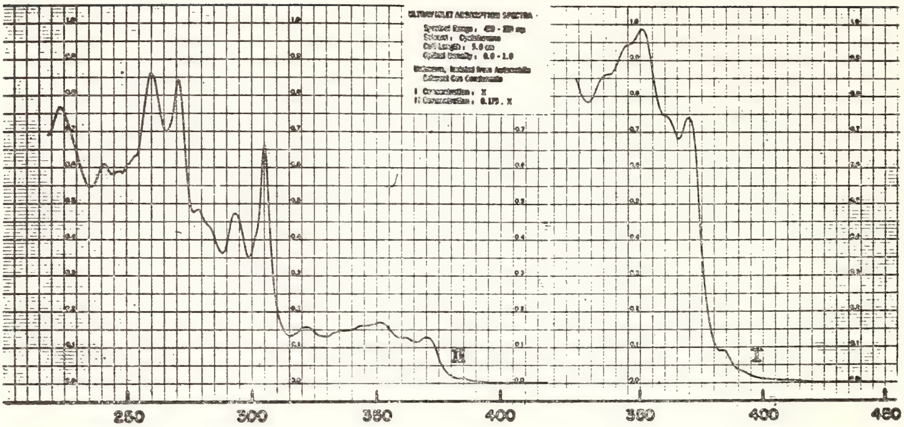
TEXT-FIGURE 4.—Ultraviolet absorption spectra of indeno[1,2,3-*cd*]pyrene and unknown.

TABLE 2.—Melting and mixture melting points of isolated hydrocarbons*

Isolated hydrocarbon	Melting point (°C)	Mixture melting point (°C)	Melting point of reference (°C)
Pyrene	153–155	152–154	154–155
Chrysene	252–255	252–254	254–256
Benzo[*mno*]fluoranthene	146–148	146–148	148–149
Benzo[*e*]pyrene	175–177	175–177	178–179
Indeno[1,2,3-*cd*]pyrene	159–160	159–162	163–164

*Melting points obtained with calibrated thermometer.

Tables 3 through 6 summarize the analytical data obtained for gasoline engine exhaust from a "city driving" run at the mileage interval of 8,000 to 12,000 miles. The data in the last 3 columns represent only the amount



Text-figure 5.—Ultraviolet absorption spectra of unknown.

of isolated aromatic hydrocarbon found and are not absolute values Such absolute values can be obtained only with the help of the tracer technique (*5*). In this technique a C^{14}-labeled hydrocarbon is added to the test sample prior to the analytical procedure. The concentration of the isotopic hydrocarbon to be added is determined by a pilot analysis of the sample. This procedure results in a paper chromatography band containing both the C^{14}-labeled hydrocarbon and the original hydrocarbon from the test material. The ultraviolet absorption spectrum represents the total amount of isolated hydrocarbon, and we determined the amount of isolated tracing compound by counting the β-radiation. In this way we were able to calculate the absolute amount of the polynuclear aromatic hydrocarbon in the test sample within a maximal deviation of ± 5 percent. To our knowledge, this is the most accurate method for the determination of traces of polycyclic hydrocarbons in combustion materials. It would be cumbersome to synthesize all hydrocarbons so far known in combustion products as C^{14}-labeled compounds. Therefore, we tried to modify the tracer technique for tritiated hydrocarbons. They can be easily obtained by the Wilzbach tritiation method and can be counted by the liquid scintillation technique (*8*).

In our analytical work so far we have worked mainly with benzo[*a*]pyrene-6-C^{14} and benz[*a*]anthracene-9-C^{14}. Despite equal precautions during the analyses we found a notably higher degree of degradation of the polynuclear hydrocarbons in gasoline exhaust than in tobacco smoke products (*5*).

In column 3 of tables 3 through 6 data are given for the relative activity of the hydrocarbons on mouse epidermis. These results are all based on data from our own laboratory where the individual hydrocarbons, in at least 3 concentrations, were tested on groups of 20 mice each. All hydrocarbons were purified by column chromatography on alumina or "Florisil"; their purities were determined by spectra, R_F values, as well as melting points.

In 1959 we reported for the first time the tumor activity of the 4 benzofluoranthenes: benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, and benz[*mno*]fluoranthene (*9*). The first 2 gave a moderate tumor activity to mouse epidermis (text-fig. 6). Recently we found a slight tumor activity for indeno[1,2,3-*cd*]pyrene (*10*). Several authors in the air pollution field have stated that dibenzo[*a,l*]pyrene is a strongly active carcinogen. A thorough study reveals that this compound was tested only once, in 1937 by Bachmann *et al.* They found that, of 2 mice tested, 1 died at the end of 182 days and the other lived for 262 days and developed 1 epithelioma (*11*). In this connection we need to recall that, according to the Food Protection Committee of the National Academy of Sciences, a component is regarded as carcinogenic only when a minimum of 3 animals is tested and a 100 percent tumor incidence is reported (*12*). Text-figure 7 shows the carcinogenic activity produced by dibenzo[*a,l*]pyrene synthesized in our laboratory (*13*). In a dose-response study we found that the tumor potency of dibenzo[*a,l*]pyrene was between that of benzo[*a*]pyrene and dibenz[*a,h*]anthracene.

At present we are engaged in developing a short-term analytical method for the quantitative determination of *N*-heterocyclic hydrocarbons, some of which have been described as carcinogenic in animal tests (*14*). A large number of the polynuclear aromatic hydrocarbons as well as the *N*-heterocyclic hydrocarbons are regarded as a major group of initiating carcinogens in combustion materials such as automobile exhaust gas. The initiating, promoting, and summation effects as well as the competitive action and retardation effects of agents in such complex materials warrant a separate study, which can be undertaken only step by step. However, we do know more about some groups of constituents and their role in experimental carcinogenesis than about others.

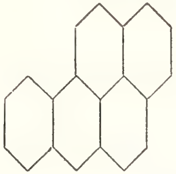
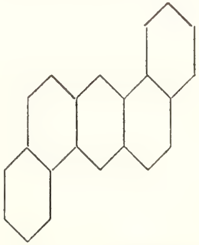
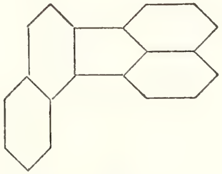
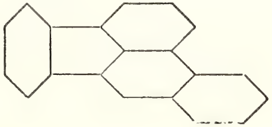
Many phenolic compounds are promoting agents that accelerate tumor formation (*10*, *15*) even for weak carcinogens (text-fig. 8). Studies in our laboratory have shown that phenol and some of its derivatives strongly inhibit cilia movement. This finding accentuates further the significance of phenol in the human environment (*16*).

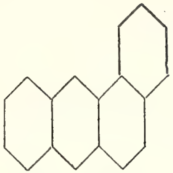
Since the standard gasoline also used for our test run contains a certain percentage of aromatic hydrocarbons (mainly benzene, toluene, and xylenes), it is possible that these are precursors of phenols formed during combustion. We therefore analyzed the automobile exhaust condensate for phenol and phenol derivatives.

For this determination we employed a technique developed for the quantitative determination of phenols in tobacco smoke (*17*). This method is based primarily upon separation by gas-liquid chromatography of phenol concentrates into individual phenols and their quantitative determination with an internal standard. Di-*n*-octyl sebacate or polyethylene glycol compound 20M (Carbowax) was used as the liquid phase, "Gas Chrom P" as support, and helium as carrier gas. Best results were achieved at 180° C (text-figs. 9 and 10).

The results of the analysis for phenols in the benzene extract of a

TABLE 3.—Polynuclear aromatic hydrocarbons isolated from gasoline-engine exhaust extract (5.023 g)

Hydrocarbon	Formula	Biological activity*	Isolated from fraction	μg	ppm	μg per 1 minute run
Benzo[*a*]pyrene		+++	14	158. 1	31. 5 (72. 6 ± 3. 6†)	2. 2 (5. 0†)
Dibenz[*a,h*]anthracene		+++	18	11. 6	2. 3	0. 16
Benzo[*j*]fluoranthene		++	14, 16	86. 6	17. 2	1. 2
Benzo[*b*]fluoranthene		++	13, 14	332	64. 1	4. 4

Benz[*a*]anthracene		+	10, 11	310	61. 7 (131. 4 ± 6. 5†)	4. 2 (9. 0†)

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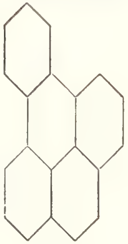
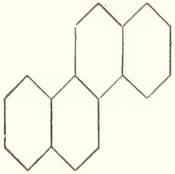
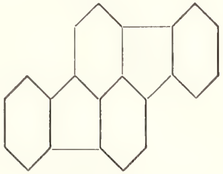
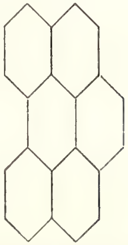
*Relative activity on mouse epidermis: +++ = active; ++ = moderate; + = weak.

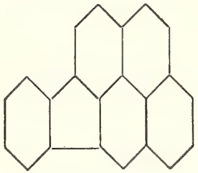
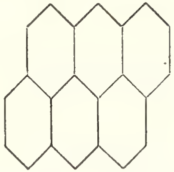
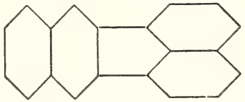
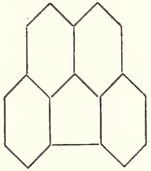
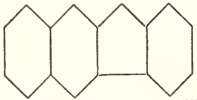
†Actual amount in the extract calculated by tracer technique.

Table 4.—Polynuclear aromatic hydrocarbons isolated from gasoline-engine exhaust extract (5.023 g)

Hydrocarbon	Formula	Biological activity*	Isolated from fraction	μg	ppm	μg per 1 minute run
Benzo[*e*]pyrene		+	13, 14	2, 120	422. 0	29. 0
Chrysene		+	13, 14	880	175. 0	12. 0
Alkylbenzo[*a*]pyrene		?	14	5. 0	1. 0	0. 07
Alkylbenz[*a*]anthracene		?	12	10	2. 0	0. 14
Alkylchrysene		?	13, 14, 15	334	66. 5	4. 6
Indeno[1,2,3-*cd*]fluoranthene		?	17	13. 3	2. 6	0. 18
Benzo[*ghi*]perylene		–	12, 13, 14	255. 0	50. 8	3. 5

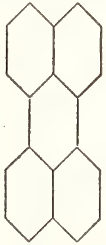
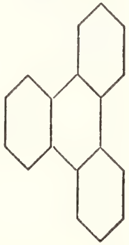
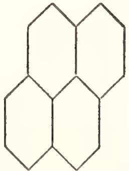
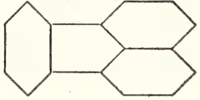
*Relative activity on mouse epidermis: + = weak activity; ? = unknown; – = inactive.

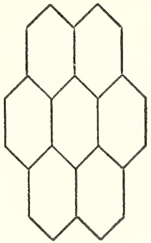
TABLE 5.—Polynuclear aromatic hydrocarbons isolated from gasoline-engine exhaust extract (5.023 g)

Hydrocarbon	Formula	Biological activity*	Isolated from fraction	μg	ppm	μg per 1 minute run
Indeno[1,2,3-*cd*]pyrene		+	17	414	82. 4	5. 7
Anthanthrene		—	15	223	44. 4	3. 1
Benzo[*k*]fluoranthene		—	16, 17	270	53. 8	3. 7
Benzo[*mno*]fluoranthene		—	7, 8, 9	750	149. 0	10. 2
11*H*-benzo[*b*]fluorene		—	6, 11	254	50. 6	3. 5

*Relative activity on mouse epidermis: + = weak activity; — = inactive.

Table 6.—Polynuclear aromatic hydrocarbons isolated from gasoline-engine exhaust extract (5.023 g)

Hydrocarbon	Formula	Biological activity*	Isolated from fraction	µg	ppm	µg per 1 minute run
Perylene		—	11	Trace	Trace	Trace
Triphenylene		—	12	220	44. 0	3. 0
Pyrene		—	3, 4, 5	8, 880	1, 770	122. 0
Alkylpyrene		?	13	40	8	0. 06
Fluoranthene		—	5, 6	4, 880	972	60. 0
Alkylfluoranthene		?	6–11	1, 312	261	17. 9

Coronene		—	15, 16	1, 692	338	23. 2

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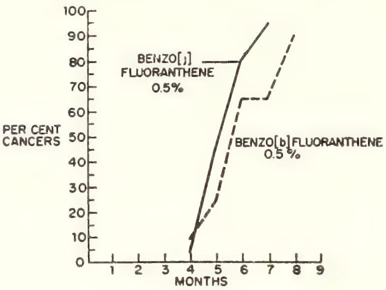
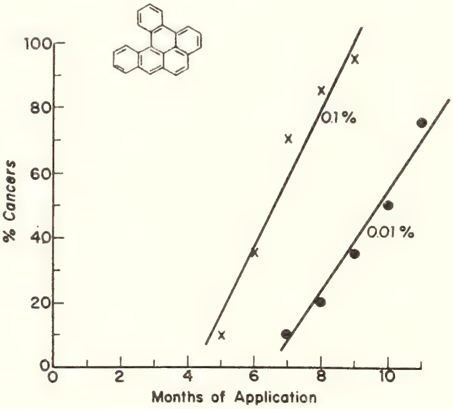
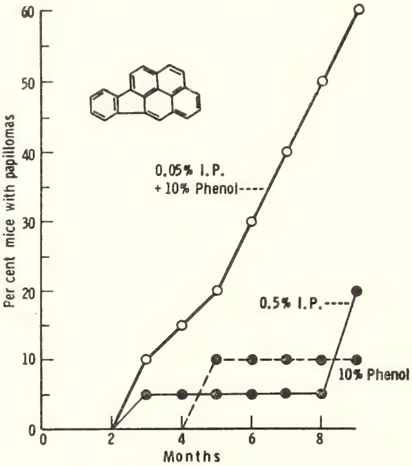
*Relative activity on mouse epidermis: — = inactive; ? = unknown.



Text-figure 6.—Tumor activity of benzo[*j*]fluoranthene and benzo[*b*]fluoranthene in 0.5 percent concentration.

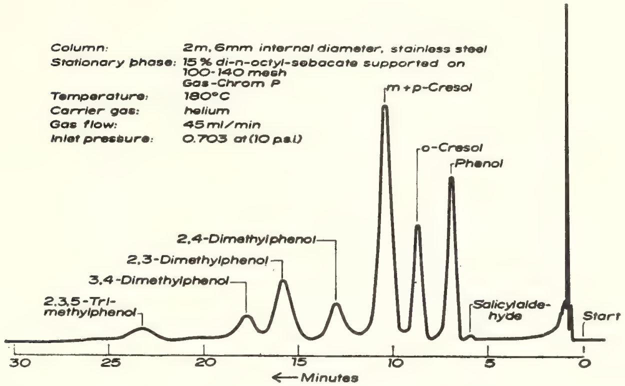


Text-figure 7.—Carcinogenicity of dibenzo[*a,l*]pyrene on mouse epidermis.

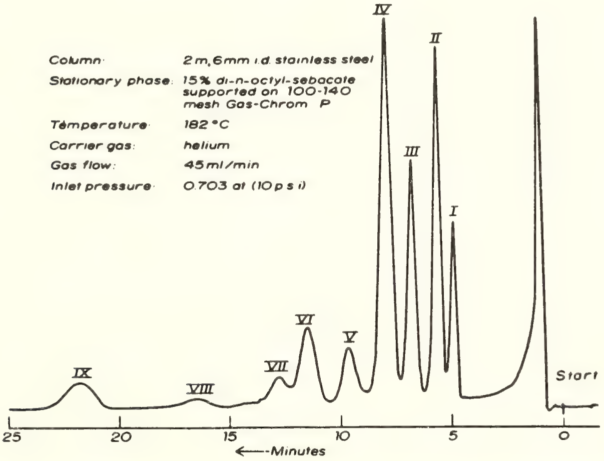


Text-figure 8.—Initiating activity of indeno[1,2,3-*cd*]pyrene.

gasoline-engine exhaust condensate are given in table 7. In brief, the analysis of the benzene extract of the gasoline-engine exhaust condensate showed 10.5 percent phenols and approximately 2 percent polynuclear



TEXT-FIGURE 9.—Chromatogram of phenol concentrate of automobile-exhaust gas condensate.



TEXT-FIGURE 10.—Chromatogram of phenol concentrate of automobile-exhaust gas condensate with two tracing compounds (I and IX).

aromatic hydrocarbons, of which less than one tenth is carcinogenic to mouse epidermis. Comparing our analysis with that of cigarette-smoke condensate (tables 8 and 9), we expected a significantly higher tumor activity with the gasoline exhaust extract. We set up parallel tests with standard cigarette-smoke condensate and gasoline exhaust extract. Both test materials in acetone solutions of 50, 33, 25, 10, and 5 percent concentrations were applied to the shaved backs of female Swiss mice 3 times weekly for 15 months. Except for the 50 percent concentration, the toxicity of both materials (survival rate and weight curve) was comparable. The 50 percent exhaust extract showed a higher toxicity than tobacco-smoke condensate in the same concentration.

TABLE 7.—Quantitative determination of individual phenolic compounds in benzene extract of gasoline engine exhaust

Compound	Amount present
Salicylaldehyde	Trace
Phenol	1. 7%
o-Cresol	1. 9%
m + *p*-Cresol	3. 0%
2,4-Dimethylphenol	1. 05%
2,3-Dimethylphenol	1. 45%
3,4-Dimethylphenol	0. 65%
2,3,5-Trimethylphenol	0. 4%

TABLE 8.—Polynuclear aromatic hydrocarbons isolated from gasoline engine exhaust and from cigarette smoke

Hydrocarbon	Biological activity[*]	ppm Isolated from exhaust concentrate	ppm Isolated from cigarette-smoke condensate
Benzo[*a*]pyrene	+++	31. 5 (72. 6 ± 3. 6[†])	0. 7 (1. 2 ± 0. 1[†])
Dibenz[*a,h*]anthracene	+++	2. 3	0. 1–0. 15
Benzo[*j*]fluoranthene	++	17. 2	0. 15–0. 2
Benzo[*b*]fluoranthene	++	64. 1	0. 1
Benz[*a*]anthracene	+	61. 7 (131. 4 ± 6. 5[†])	0. 1
Benzo[*e*]pyrene	+	422. 0	0. 1
Chrysene	+	175. 0	1. 5–2. 0
Alkylbenzo[*a*]pyrene	?	1. 0	P[‡]

[*] Relative activity on mouse epidermis: +++ = active; ++ = moderate; + = weak; ? = unknown.

[†] Actual amount in the tested material calculated by tracer technique.

[‡] P indicates that the hydrocarbon was present but not calculated.

TABLE 9.—Some phenolic compounds isolated from gasoline-engine exhaust concentrate and from cigarette-smoke condensate[*]

Compound	Relative promoting activity[†]	Exhaust gas concentrate (%)	Cigarette-smoke condensate (%)
Phenol	++	1. 7	0. 3
o-Cresol	++	1. 9	0. 08
m + *p*-Cresol	++	3. 3	0. 25
2,4-Dimethylphenol	++	1. 0	0. 1

[*] In addition, smaller amounts of phenolic compounds were identified in: (*a*) exhaust gas concentrate: salicylaldehyde (−); 2,5-dimethylphenol (?); 3,5-dimethylphenol (++); 2,3,5-trimethylphenol (?). (*b*) Cigarette smoke: salicylaldehyde (−); guaiacol (?); ethylphenols (+); 3,5-dimethylphenol (++).

[†] Relative promoting activity according to Boutwell and Bosch (*15*).

Figure 1 shows a group of 4 mice that were treated with 25 percent gasoline exhaust extract for 9 months. Two showing papilloma and two carcinoma typify results obtained throughout this study. In general the appearance of papilloma and cancer occurred several weeks earlier with gasoline exhaust extracts than with tobacco-smoke condensate (table 10). The conversion time from papilloma to carcinoma is relatively long for both materials in comparison to the conversion time observed with known carcinogenic aromatic hydrocarbons. For polynuclear hydrocarbons conversion generally was observed within a few weeks. A comparison of tumor potency, according to Blanding's method (*18*), showed the exhaust

TABLE 10.—Occurrence of papilloma and carcinoma in mice after treatment with gasoline exhaust extracts and tobacco-smoke condensate*

Gasoline exhaust and condensate (%)	ICR mice	Date begun	Months																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Exhaust 50	50	8–26–60	50	50	50	**1**–50	**5**–50	**7**–50	**10**–49	**23**–47	**30**–(40)–*2*	**37**–28–*4*								
Tobacco-tar control 50	50	8–26–60	50	50	50	50	49	49	**1**–49	**4**–47	**6**–46	**8**–42								
Exhaust 33	50	12–23–59	50	49	49	48	48	48	48	**4**–46	**9**–(45)–*1*	**11**–43–*1*	**17**–34–*2*	**21**–33–*3*	**23**–30–*6*	**30**–21–*11*	**30**–17–*12*	**30**–12–*15*	**30**– 2–*24*	**30**– 2–*27*
Tobacco-tar control 33	50	12–23–59	50	49	47	44	**1**–44	**2**–44	**3**–44	**4**–43	**5**–41	**7**–40	**10**–39	**11**–(36)–*1*	**17**–33–*2*	**7**–32–*2*	**17**–26–*2*	**17**–22–*3*	**17**–14–*8*	**17**– 9–*10*
Exhaust 25	50	12–23–59	50	50	49	49	47	45	44	**2**–4–(*1*)	**5**–43–*2*	**9**–40–*2*	**11**–38–*3*	**19**–35–*4*	**21**–33–*6*	**27**–33–*6*	**27**–27–*9*	**30**–21–*14*	**30**– 9–*21*	**30**– 9–*24*
Tobacco-tar control 25	50	12–23–59	50	50	50	49	48	**1**–48	**1**–47	**2**–47	**3**–46	**4**–46	**4**–46	**4**–41	**8**–39	**9**–38	**9**–32–(7)	**9**–28–*1*	**9**–24–*4*	**9**–19–*11*
Exhaust 10	50	12–23–59	50	50	50	50	50	49	48	48	**2**–47	**2**–46	**2**–(42)–*2*	**4**–42–*2*	**11**–37–*4*	**19**–34–*5*	**24**–31–*6*	**24**–26–*7*	**25**–17–*15*	**25**–13–*16*
Tobacco-tar control 10	50	12–23–59	50	50	48	47	46	46	45	45	45	44	44	43	**1**–38	**2**–37	**2**–36	**2**–27	**3**–24	**3**–18
Exhaust 5	50	12–23–59	50	49	48	47	47	47	46	45	45	**1**–44	**1**–43	**1**–43	**1**–41	**2**–41	**2**–39	**2**–31	**2**–31	**2**–25
Tobacco-tar control 5	50	12–23–59	50	50	49	46	46	43	44	42	41	41	39	46	35	30	28	25	25	19

*Numbers in arabic = No. of surviving animals; numbers in boldface type = No. of papillomas; numbers in italics = No. of carcinomas; figures in parentheses = first appearance of carcinoma.

"tar" had almost twice the tumor potency of cigarette-smoke condensate. Despite the fact that cigarette-smoke condensate contains other known tumorigenic and tumor-promoting agents such as polyphenols and long-chain fatty acids, the results of this comparison are surprising, when judged on the basis of the chemical analysis. These results again reveal the difficulty in accounting for the tumorigenic activity of a mixture of this kind.

Steiner and Falk (*19*), as well as Falk and Kotin (*20*), found some inhibiting effects of related carcinogenic hydrocarbons when such compounds were applied together subcutaneously. This may be one possible explanation for the relatively low tumor potency of exhaust extract when compared with tobacco-smoke condensate. At present we are studying the effect of this phenomenon on mice treated with the compound cutaneously. Our early results indicate the inhibiting effect of phenanthrene on benzo[*a*]pyrene; fluoranthene and pyrene appear to accelerate the tumor potency of benzo[*a*]pyrene (*10*). These latter results, however, need confirmation by repeated experiments, which are being carried out in various dilutions.

Further, we consider it important to analyze automobile-exhaust gas condensate for long-chain aliphatic hydrocarbons, since some of them proved to have an inhibiting effect on the resorption of carcinogenic compounds (*10*). Other important aspects in the study of automobile exhaust gas are the operation time of an engine and the different types of fuel used. Our results in this regard are summarized in tables 11 and 12. Table 11 shows the increase of benzo[*a*]pyrene as a possible function of the mileage of the test engine. Table 12 shows the amounts of phenol and benzo[*a*]pyrene in the exhaust of an engine operated with standard gasoline, with diisobutylene, and with isooctane.

TABLE 11.—Amounts of benzo[*a*]pyrene at various mileage intervals with a V-8 engine, "city driving," "standard" gasoline

Mileage interval	Benzo[*a*]pyrene per minute run (μg)
8, 000–12, 000	5. 0
19, 300–25, 500	6. 2
25, 500–29, 000	9. 0
29, 000–33, 200	27*

*This value cannot be regarded as representative; (malfunction of the engine).

TABLE 12.—Amounts of benzo[*a*]pyrene and some phenols per minute run in the exhaust of an engine operated with different fuels

Type of fuel	Mileage interval	Benzo[*a*]-pyrene (μg)	Phenol (μg)	*o*-Cresol (μg)	*m* + *p*-Cresol (μg)
Standard gasoline	19, 300–25, 500	6. 2	1, 350	1, 400	2, 750
Standard gasoline	25, 500–29, 000	9. 0	1, 400	1, 600	2, 700
Isooctane*	0–874	2. 5	13	Trace	Trace
Diisobutylene	875–1, 748	0. 7	59	51	78

*Before this run the engine was completely overhauled.

These results indicate, as did the studies by Badger *et al.* (*21*), that almost all organic matter, to a certain extent, forms polynuclear aromatic hydrocarbons upon pyrolysis. These data also show clearly that specific precursors for polycyclic hydrocarbons and for phenols reside mainly in the aromatic portion of standard gasoline. Further studies in this direction are planned, including the complete analysis of crankcase blowby material. In a previous analysis, a considerable amount of polycyclic hydrocarbons was found in the crankcase oil.

To determine the extent to which automobile exhaust contributes to general air pollution, detailed analyses of air pollution samples from various cities and rural areas are under way in cooperation with the Research Laboratories of the General Motors Corporation.

CONCLUSIONS

The benzene-soluble matter of the gasoline-engine exhaust condensate of a V-8 engine operated on a "city driving schedule" was analyzed for polynuclear aromatic hydrocarbons and phenols. Twenty-five known and 2 unknown polynuclear aromatic hydrocarbons were isolated by column and paper chromatography and identified by R_F values and ultraviolet absorption and fluorescence spectra. Six hydrocarbons were isolated in crystalline form. Nine of the isolated polynuclear aromatic hydrocarbons were confirmed or proved to be carcinogenic in the animal test. Eight phenols were identified in the exhaust gas condensate; 5 of them are known to be tumor-promoting agents to mouse epidermis.

The analyzed material was applied to mouse skin in 5 different concentrations and its tumor activity was compared to that of corresponding concentrations of cigarette-smoke condensate. It was found that the tumor potency is about 2 times higher for the exhaust "tar" than for the cigarette-smoke condensate. An attempt is made to interpret this finding in terms of chemical constituents.

It should be emphasized that these laboratory studies merely provide an understanding of the factors involved in air pollution carcinogenesis. In the final instance, they can only be fully interpreted in studies involving man. Despite this fact, we regard any effort which may lead to a reduction of carcinogens and cocarcinogens in exhaust fumes as most pertinent.

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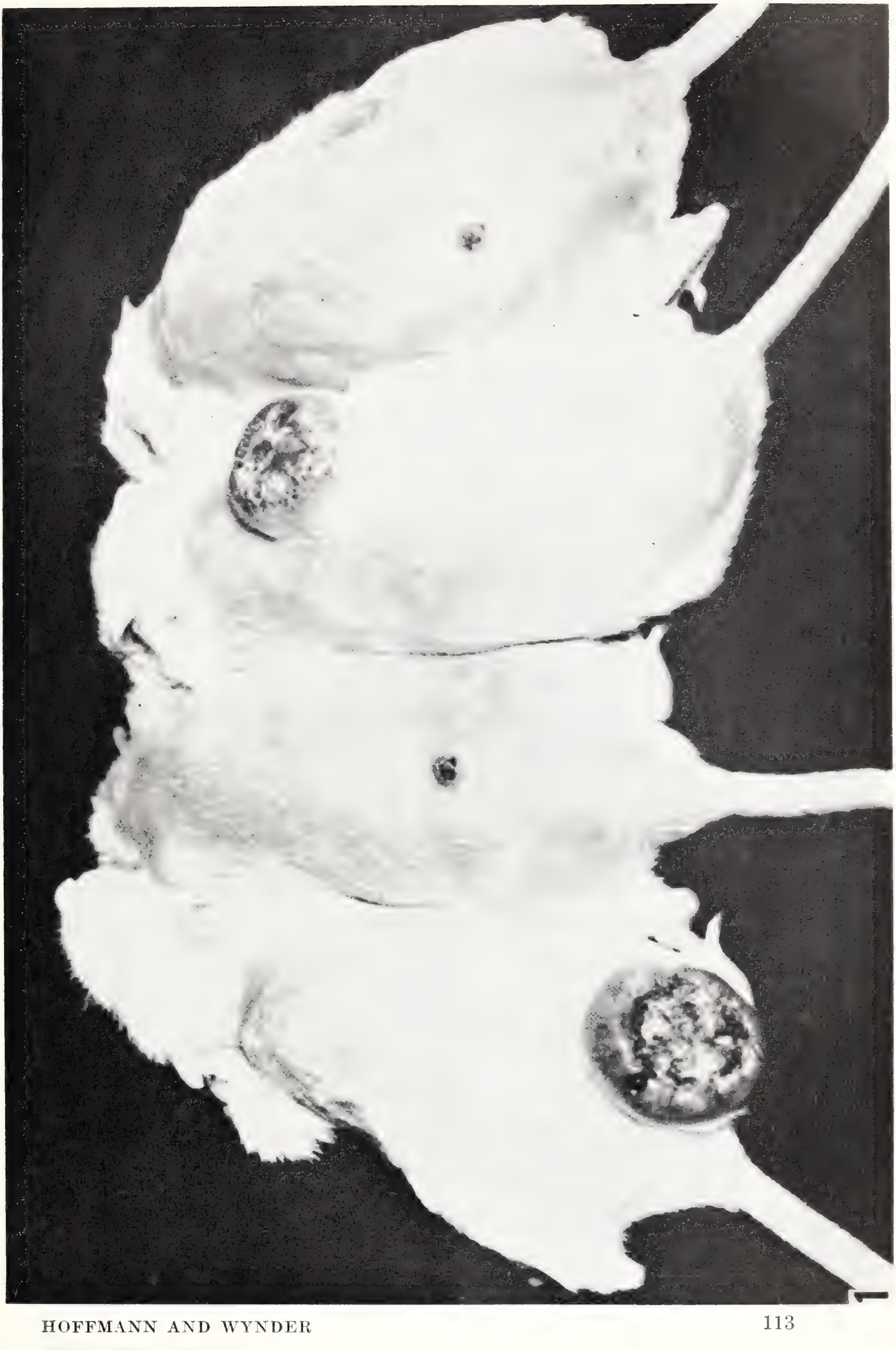
PLATE

PLATE 12

FIGURE 1.—Group of 4 mice treated with 25 percent gasoline exhaust extract for 9 months.



HOFFMANN AND WYNDER

DISCUSSION

DR. COMMINS: I would like to make some observations on work we have done in London. In British gasoline, considerable amounts of polycyclic aromatic hydrocarbons are present to the extent of up to 2.4 μg of pyrene per ml of gasoline, and up to 0.4 μg per ml for benzo[*a*]pyrene. Would Dr. Hoffmann or anybody else like to comment about American gasoline? I feel this may be the reason for the presence of some of these compounds in the exhaust.

DR. HOFFMANN: No, we have not done this. We have not tested gasoline, since we were interested more in the exhaust itself.

DR. FALK: We have investigated the presence of polycyclic hydrocarbons in one type of gasoline. We did not find any benzo[*a*]pyrene.

DR. COMMINS: I would like to comment a little further. In Britain we have what we call commercial grades and higher grades; the latter contain added aromatic fractions. In some of the commercial grades we detected only minute traces of these hydrocarbons, but in the aromatized fuels we have detected them. It is possible that a great amount of the aromatic hydrocarbons in the exhaust may come from the fuel itself. In one experiment with a fuel containing no aromatic hydrocarbons and another similar fuel that did contain some, we obtained a reduction of the polycyclic aromatic hydrocarbon content of the exhaust. This is rather peculiar and I do not understand it. The concentration of hydrocarbons in the smoke was the same in both instances, but with the fuel containing the polycyclic aromatic hydrocarbons there was a one-third decrease in total polycyclics.

DR. KATZ: I would like to ask Dr. Hoffmann whether he found coronene, because coronene has been found consistently in our work on air pollution samples. Another question is this: In connection with your work on the exhaust gases of gasoline engines, did you find some correlation between the amount of smoke generated in the exhaust and the concentration of polynuclear aromatic hydrocarbons?

DR. HOFFMANN: You will have to ask Mr. Begeman about this.

MR. BEGEMAN: In our experiments we do not see the exhaust gas visually to know whether there is more smoke or not.

DR. KATZ: I think this is a very important point, because one of the ways of controlling polycyclics from exhaust gases is to cut down on the production of visible smoke. I believe that the concentration of polynuclear hydrocarbons in a smoke is proportional to the density of the smoke. In other words, if you cut down on smoke production then you automatically reduce the polycyclic hydrocarbons.

DR. HOFFMANN: I think if we would drive our cars with diisobutylene we would significantly reduce the amount of phenols as well as polynuclear aromatic hydrocarbons in the exhaust. But this is a budget question and we may not be able to afford that. I think the figures of Dr. Commins show that in diesel engines there is a lower amount of polynuclear hydrocarbons present than in gasoline engine exhaust. But when you drive behind a bus and see the large quantity of smoke pouring out, there is no doubt that the bus diesel engines let out more exhaust smoke than the gasoline engines. There is no absolute figure. However, we have only 6 or 7 runs so far, and we do not like to make any conclusion from this.

DR. LINDSEY: I should like to ask Dr. Hoffmann if the cancer-producing activity of the fraction which he isolated corresponded quantitatively with the amounts of carcinogenic hydrocarbons discovered by quantitative analysis.

DR. HOFFMANN: It is too early to draw any conclusion from our work.

DR. FALK: We have investigated the correlation between polycyclic aromatic hydrocarbon concentration and the amount of smoke. We manipulated a diesel engine so as to produce more or less soot. The more soot we got, the more polycyclics we found. We actually managed to get this diesel engine to work so that there was absolutely no soot whatsoever coming out under a full load. Under these conditions we found no benzo[*a*]pyrene.

DR. KATZ: I can corroborate the remarks of Dr. Falk. We have had the opportunity of collecting samples from a 1,750-horsepower railway diesel. This is a huge engine that has been set up on an experimental basis at the National Research Council of Canada in Ottawa, Canada. We have been tied into the project, and we confirmed the findings of Dr. Commins and his group that a properly operated diesel will give you far less carcinogenic or polynuclear aromatics than a gasoline engine In fact, as Dr. Falk said, there are times when you get few, if any, aromatics. Most of the material collected when the engine is idling is mainly unburned diesel oil. I think the main difference between the two types of engines is that the diesel operates on a high-temperature cycle, whereas the gasoline engine operates on a comparatively low-temperature cycle.

CHAIRMAN SAWICKI: I would like to bring in one point. Are you trying to say that the amount of polycyclic hydrocarbon in the particulate is proportional to the amount of particulates?

DR. KATZ: There is no correlation between the smoke or soot coming out of an engine and the total polynuclear hydrocarbons. A great deal of this may consist of pyrene and other compounds, so I would not care to relate it to benzo[*a*]pyrene. I have not seen any published work as specific as that, but I think it is related and ties in with the findings of Commins and his group that there is a relation between smoke production of a diesel and the polynuclear aromatic fraction.

DR. COMMINS: I would not quite agree with Dr. Katz on this point. We have studied recently the pollution in road tunnels under the River Thames in London and have found little correlation between smoke and polycyclic aromatic hydrocarbons. One can control diesel engines so that they produce very low concentrations of hydrocarbons, yet still emit vast quantities of smoke. The worst condition I found was in following vehicles traveling uphill north of London. These lorries were emitting vast clouds of smoke. This was on a hill about a mile long, and we collected smoke from a sampler attached to a private car. We collected about 10 mg of smoke. The concentration of benzo[*a*] pyrene in that smoke was no greater than 25 μg per g, which is pretty low in comparison with most sources of benzo[*a*]pyrene, yet we had a lot of smoke. When you maintain diesel vehicles such as London Transport's in good condition, the concentration of benzo[*a*]pyrene can be as low as 1 or less μg per g.

CHAIRMAN SAWICKI: We have done some analysis of the particulates from auto exhaust, and we could not get any correlation between weight of particulate and amount of the polycyclic hydrocarbons present.

DR. KATZ: I think this question is tied in with what you mean by smoke. I mentioned before that you can get virtually unburned oily products which do not contain polynuclears, mostly composed of the original oil or fuel. However, if you refer to smoke as the incomplete soot product, that is another matter. We have to define our terms.

MR. BEGEMAN: I would like to add a comment to Dr. Hoffmann's remark in regard to the burning of diisobutylene. In an experiment that we carried out in our engine, which Dr. Hoffmann reported on, the diisobutylene fuel was burned without lead. This is another factor which we think may be significant. The commercial gasoline used had the usual amount of lead, about 3 ml per gallon. Further studies are being carried out with various loads, and we think that factor might be significant also in the amount of polynuclear aromatics emitted.

DR. BILLER: I think I can add perhaps just a little information to this question of a possible relationship between polynuclear aromatics and the amount of soot coming from an automobile engine. I think we ought to emphasize that there is quite a difference between the mechanisms by which you produce smoke or soot in a diesel and an automobile engine. In a diesel the smoke results usually from a malfunction; in an automobile engine, even a normally functioning engine can emit soot. This is dependent further on the fuel ratio. The more fuel you have for the amount of air, the more soot you will get. Several years ago we did some research in this. It was not a very extensive study, so these are rather preliminary results and we have never recorded them. But we did find that if we ran an engine at different air-fuel ratios essentially under no-load conditions, the total amount of polynuclear aromatics was approximately proportional to the organic fraction that was left behind after acetone extraction. Under lean conditions of operation, not much polynuclear aromatic material could be expected in the exhaust. We also found polynuclear aromatics in exhaust when isooctane was used as a fuel, whether or not it had lead in it. Again this study was not extensive enough to really point up any differences.

DR. THOMAS: This all ties in with the general picture of combustion, which you can find scattered throughout the literature. If you start with pyrolysis and then increase the amount of oxygen going through the self-sustaining combustion or autoxidation region up to stoichiometric conditions and beyond, when you come out of the pyrolytic region you have a large amount of soot produced and a relatively large amount of the condensed polynuclear aromatics. As you get closer to the stoichiometric conditions, soot decreases and the condensed polynuclear aromatics also decrease, consistent with the point brought out by Dr. Katz. But when you study the over-all picture, you have to consider internal combustion and separate the auto from the diesel. Then you have to consider external-type combustion, where you are burning at atmospheric pressure, as a completely different topic.

DR. FALK: I would like to make one more comment that raises a question in relation to everything else that was said. About 10 years ago I studied the difference in the output of various polycyclic aromatic hydrocarbons in the exhaust of an engine under different load conditions. We compared pyrene, fluoranthene, benzo[*a*]pyrene, benzo[*e*]pyrene, coronene, anthracene, and benzperylene. Although the pattern is usually the same if you compare at any time the ratios of all these compounds to each other, they do vary. So there is a certain amount of load which will produce less pyrene and more benzo[*a*]pyrene. All these correlations are roughly correct, if you increase the load in the gasoline engine. At lower loads different results are obtained at different times. It is a rather confusing situation.

DR. HOFFMANN: Dr. Falk, we are very well aware of your work, and I think this was the reason that General Motors Research Laboratories has operated the engine at all times under the same conditions of load and so on. This way we obtain meaningful results.

DR. ALTSHULLER: I hope we are not losing sight of the fact that the automobile or diesel is just a test vehicle that we can operate only under certain specific conditions. But you cannot control the loading of material going into the atmosphere from millions of automobiles. Furthermore we have to remember that the atmosphere is ventilating at a great rate. And finally many of these compounds, as will be indicated, are undergoing photooxidation reaction in the atmosphere. Much previous work and some of the work which we will report next week shows that this is true for most of the gases of the organic species in the atmosphere. They are not stable in the atmosphere, so the relative concentration that you find when you condense directly from the exhaust may not represent the relative concentration of the material in the atmosphere.

DR. HOFFMANN: We are not attempting to draw any relationship between air pollutants and health or specifically lung cancer from these data. However, these studies indicate that we can reduce significantly the amount of polynuclear aromatic hydrocarbons in the exhaust of cars when certain fuels are used. As often in the history of medicine (Walter Reed and Ignaz Semmelweis, for example), prevention can precede the understanding of a disease.

Decomposition of Polycyclic Aromatic Hydrocarbons Under Laboratory Illuminations[1]

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SUMMARY

A study was undertaken to determine whether decomposition of polycyclic aromatic hydrocarbons occurs under the ordinary conditions of laboratory illumination. Benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene were dissolved in purified cyclohexane and irradiated by ultraviolet light. After similar irradiation periods of roughly 40 hours, 25 percent of the benzo[*a*]pyrene and 84 percent of the 7,12-dimethylbenz[*a*]anthracene had decomposed. Fourteen aromatic hydrocarbons were irradiated by daylight-type fluorescent lamps. Among the hydrocarbons tested, naphthacen was most sensitive: 47 percent had decomposed after 41 hours of irradiation. No other compounds showed appreciable decomposition after 80 hours of irradiation. The effects of other types of illumination and solvent were also examined.—NCI Monogr. No. 9: 117–133, 1962.

IT IS a well-known fact that polycyclic aromatic hydrocarbons are not stable under ultraviolet irradiation (*1*, *2*). Some of these hydrocarbons are drastically decomposed by irradiation; therefore, great caution must be exercised in a laboratory to prevent such photochemical degradation. Although a laboratory may be dimly illuminated or illuminated with umber-colored lamps, hydrocarbons are irradiated by ultraviolet light generated by a mercury lamp during fluorescent examination. Very little was known about the magnitude of the photochemical decomposition of these hydrocarbons under laboratory illuminations.

DECOMPOSITION OF AROMATIC HYDROCARBONS UNDER DAYLIGHT-TYPE FLUORESCENT LAMPS (EXPERIMENT 1)

In recent years fluorescent lamps have been used to replace incandescent lamps for illumination. Fluorescent lamps emit ultraviolet light, which may affect polycyclic aromatic hydrocarbons. The hydrocarbons examined in this experiment are listed in table 1. They were purified chromatographically, and each of the purified hydrocarbons was dissolved in purified cyclohexane or dichloromethane. The resulting solutions had absorbances of 1 to 2 at their main absorption peaks. The cyclohexane was purified by a series of treatments: first by nitration, then by passing the nitrated solvent through a column of activated silica gel, and finally by distilling the eluate. The absorbances of the purified cyclohexane were 0 down to 240 mμ, 0.2 at 220 mμ, and 0.5 at 210 mμ. Dichloromethane was purified simply by distillation. It had absorbances of 0.06 and 0.2 at 250 and 240 mμ, respectively.

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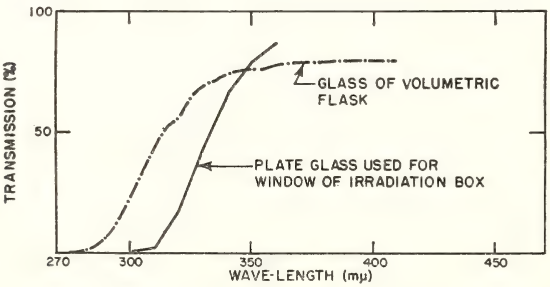
[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

An aliquot, 50 ml, of each of the solutions was taken up into 100 ml volumetric flasks, and the flasks were then sealed with silicon grease. The light transmission of the glass of the flasks is shown in text-figure 1. These flasks were placed in a dark room and uniformly irradiated by six 40-watt fluorescent lamps located 160 cm above the flasks. The lamps were of the daylight type (Matsuda FL-40,D) most popular in Japan. According to the information supplied by the manufacturer on the spectral characteristics of the lamps, they generate a relatively small but not negligible amount of energy at wavelengths less than 400 mμ (text-fig. 2). The vertical illumination near the site of the flasks, as measured by an illuminometer (Toshiba No. 5), was 1,100 Lux and the horizontal 400 Lux. As control, aliquots of each solution were taken up in flasks in the same way, but these were kept in the dark. The flasks were covered with aluminum foil and placed in the same room. The temperature in the room was 28° to 34° C. To ascertain any photochemical changes that might occur under these experimental conditions, the absorption spectra of the solutions were determined intermittently by use of a Beckman DK 2 recording spectrophotometer. The results obtained are summarized in table 1.

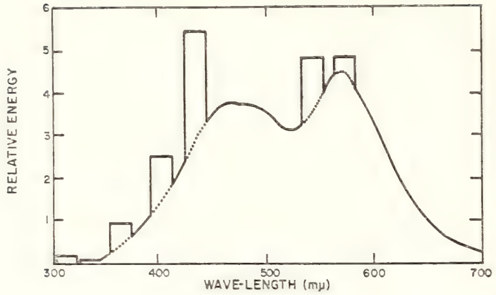


TEXT-FIGURE 1.—Light transmission of glasses through which light is entered into the solutions.

Of the hydrocarbons examined, phenanthrene, fluorene, pyrene, fluoranthene, chrysene, benz[*a*]anthracene, and benzo[*k*]fluoranthene were not changed by the illumination, whether dissolved in cyclohexane or in dichloromethane. However, the other 5 compounds decomposed. Naphthacene, as expected, was most unstable: Its characteristic absorption peaks disappeared completely after 3 days of irradiation. Whereas

Table 1.—Decomposition of aromatic hydrocarbons under illumination with fluorescent lamps of daylight type

		Test 1 Cyclohexane		Test 2 Cyclohexane			Control for test 1 and 2 (kept in dark) Cyclohexane		Test 3 Dichloromethane	Control for test 3 (kept in dark) Dichloromethane
Substance	Concentration M × 10^{-5}	After 41 hours (%)	After 83 hours (%)	After 72 hours (%)	After 235 hours (%)	After 377 hours (%)	After 235 hours (%)	After 377 hours (%)	After 115.5 hours (%)	After 115.5 hours (%)
Naphthacene	0. 78	−47	−80	−100	−100		−9		−100	−4
7,12-Dimethylbenz[*a*]anthracene	0. 74	−26	−49	−44	−75		−3		−75	0
Benzo[*a*]pyrene	1. 34	0	−1		−16	−25	0		−5	0
7-Methylbenz[*a*]anthracene	0. 95	0	0		−16	−29		0	−17	0
Anthracene		−9	−9		−12	−10		0	0	0
Dibenz[*a,h*]anthracene	1. 51	0	0		0			0	0	0
Phenanthrene	1. 58	0	0	0	0			0	0	0
Pyrene	1. 17	0	0	0	0			0	0	0
Fluorene	2. 62	0	0	0	0			0	0	0
Fluoranthene	3. 00	0	0	0	0			0	0	0
Chrysene	0. 60	0		0	0			0	0	0
Benz[*a*]anthracene	1. 31	0	0	0	0			0	0	0
Benzo[*k*]fluoranthene	1. 64	0	0	0	0			0	0	0



TEXT-FIGURE 2.—Spectral characteristics of daylight-type lamp (Matsuda FL-40,D).

benz[*a*]anthracene was stable, its alkyl derivatives, 7-methyl- and 7,12-dimethylbenz[*a*]anthracene, both with carcinogenic potency, were unstable. The latter was particularly unstable. Benzo[*a*]pyrene was also labile to the light, decreasing gradually with prolonged irradiation. Initially, anthracene seemed to be decreased in concentration by the irradiation; however, the decrease did not progress when the irradiation was prolonged, as noted in experiments 1 and 2. This peculiar observation may have been the result of an accidental leak of the vapor from the solvent during the irradiation. Such a leak, if it occurred, could have caused concentration of the solution, offsetting the decomposition. From the control experiment, it was demonstrated that naphthacene and 7,12-dimethylbenz[*a*]anthracene decreased in concentration even when kept in the dark. This decrease was much smaller than that for the irradiated solution.

The stable compounds mentioned were not altered by the irradiation, whether dissolved in cyclohexane or in dichloromethane. However, the unstable ones were unstable in both solvents. It seemed that these two solvents had somewhat different effects on the stability of the hydrocarbons, but this was not clearly demonstrated in the experiment.

DECOMPOSITION OF AROMATIC HYDROCARBONS BY SUNLIGHT (EXPERIMENT 2)

This experiment was intended to clarify whether aromatic hydrocarbons decompose when exposed to sunlight coming into a room through glass windows. Cyclohexane solutions of the 13 hydrocarbons listed in table 2 were prepared and stored in volumetric flasks in the same way as those for experiment 1. So that the exposure of the solutions to the sunlight could be maintained as uniformly as possible, a simple device was employed. The 13 flasks were placed in a circle on a disc, 50 cm in diameter, in a wooden box 75 × 55 × 60 cm. The front part of the box had a window, 65 × 45 cm, made of a 3 mm-thick plate glass, which served as the only entry for light. The inside of the box was painted black. The disc, together with the flasks on it, was slowly rotated by an electric motor. Thus, the solutions in the flasks were evenly exposed to the light entering the window. This box was placed in a room so that it faced the southeast and a window from which the glass had been removed. The sunlight coming in from the open air hit first the glass window of the box, then the glass of the flasks, and finally the solutions in the flasks. The light transmission of the glass plate used for the window of the box is shown in text-figure 1. It is calculated, therefore, that the intensity of the sunlight reaching the box was decreased by 40 percent at 350 mμ, 83 percent at 320 mμ, and nearly 100 percent at 310 mμ, before reaching the solutions. The solutions were exposed to sunlight for 5 days in July, from approximately 9 o'clock in the morning until 5 in the afternoon. The intensity of the light entering the irradiation box was measured by an illuminometer located behind the window glass. A definite hourly variation but only slight daily variation of the intensity was observed, approximately 6,000 Lux at 9 o'clock in the morning, 3,000 Lux at noon, and about 1,300 Lux at 5 in the afternoon.

TABLE 2.—Decomposition of aromatic hydrocarbons by indoor sunlight

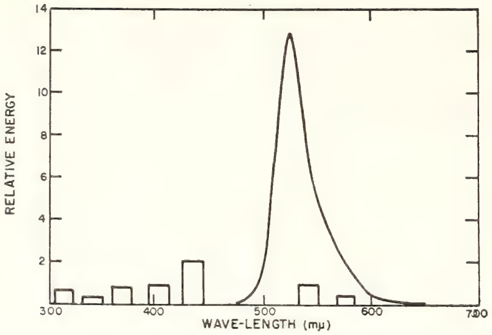
	After irradiation for 10 hours (%)	After irradiation for 45.5 hours (%)
Naphthacene	−70	−100
7,12-Dimethylbenz[*a*]anthracene	−44	−75
11 *H*-Benzo[*b*]fluorene*	−8	−31
Benzo[*a*]pyrene	−3	−12
Anthracene	−6	−12
Dibenz[*a,h*]anthracene	0	−10
Phenanthrene	0	0
Pyrene	0	
Fluorene	0	0
Fluoranthene	0	0
Chrysene	0	0
Benz[*a*]anthracene	0	
Benzo[*k*]fluoranthene	0	0

*Concentration of solution is 1.49×10^{-5} M.

As shown in table 2, the results obtained were very similar to those for experiment 1. It was noted that the decomposition occurred more quickly in experiment 2 than in experiment 1. A newly tested compound, 11 *H*-benzo[*b*]fluorene, was very labile to light.

DECOMPOSITION OF AROMATIC HYDROCARBONS UNDER GREEN FLUORESCENT LAMPS (EXPERIMENT 3)

It seemed worthwhile to examine these photochemically unstable compounds to see if they would be affected by illumination from green fluorescent lamps. Theoretically, green light does not seem to affect these compounds. Naphthacene and 7,12-dimethylbenz[*a*]anthracene were chosen for this experiment. Their solutions in dichloromethane were exposed to a yellowish-green light emitted from 6 green fluorescent lamps (Matsuda FL-40, G). The spectrum of light emitted by this type of lamp is shown in text-figure 3.



Text-figure 3.—Spectral emission of green fluorescent lamp (Matsuda FL-40,G).

The compounds tested were decomposed quickly by the yellowish-green light. Nearly all of the naphthacene and 62 percent of 7,12-dimethylbenz[*a*]anthracene were destroyed by 72 hours of exposure, contrary to our expectation. This seemed to be due to the fact that, in addition to the predominant green light, the lamps emit a fairly large amount of energy at a wavelength less than 450 mμ, which corresponds to the mercury lines. Thus, it was demonstrated that a green fluorescent lamp also decomposes some aromatic hydrocarbons.

DECOMPOSITION OF BENZO[*a*]PYRENE AND 7,12-DIMETHYLBENZ[*a*]ANTHRACENE BY IRRADIATION OF ULTRAVIOLET LIGHT (EXPERIMENT 4)

Chemical analysis of aromatic hydrocarbons usually involves frequent irradiation of the hydrocarbons by ultraviolet light generated by a mercury lamp. Therefore, it was intended to learn the magnitude of the photochemical decomposition that aromatic hydrocarbons may suffer from this irradiation. Benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene were chosen for this experiment. They were purified and dissolved in purified cyclohexane in the same manner as those hydrocarbons used in experiment 1. The solutions were each taken up into a quartz absorption cell with 1 cm light path. A thin starch was placed between the cell and its stopper to make the cell airtight. The cell was irradiated with ultraviolet light generated by a 100-watt Matsuda Super High Pressure Mercury-vapor lamp and filtered through a Corning filter #5970. Thus, the ultraviolet light used for the irradiation consisted mostly of 365 mμ and 366 mμ lines of mercury. The intensity of the light was 2.62×10^{13} quanta per second cm^2 at the surface of the cell, as determined by actinometry with uranyl oxalate (*3*).

As shown in table 3, the concentration of benzo[*a*]pyrene was decreased by irradiation, whereas no change occurred when this hydrocarbon was stored in dark. Similar but more drastic decomposition of 7,12-dimethylbenz[*a*]anthracene occurred. After 42 hours of irradiation, only two absorption peaks, 286 mμ and 297 mμ, characteristic for this compound, remained and a new peak appeared at 233 mμ. After 62 hours of irradiation, these remaining peaks disappeared and the new peak had diminished. After 130 hours of irradiation, no absorption peak was observable down to 220 mμ, which showed a complete destruction of the aromatic structure of the compound.

TABLE 3.—Decomposition of benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene by ultraviolet light

Time (hr)	Benzo[*a*]pyrene Irradiated (%)	Benzo[*a*]pyrene Stored in dark (%)	7,12-Dimethylbenz[*a*]-anthracene Irradiated (%)
17.5	−10	0	
42	−24	0	−84
62	−34	0	−100

Since it is well known that the presence of oxygen accelerates photochemical decomposition of substances, the possible effect of saturation with N_2 or O_2 on the decomposition of the benzo[*a*]pyrene solution was examined. Each solution was taken up in a quartz cell and then saturated with N_2 or O_2. The N_2 was purified by passage over reduced copper heated to 200° C; the O_2 used was 99 percent pure. The gas was introduced through a fine glass tube into the cell and allowed to flow for 5 minutes. The tube was then pulled out of the cell and the cell carefully sealed with starch before it was irradiated.

As shown in table 4, decomposition occurred even after the solution was saturated with N_2, but was markedly less when the solution was exposed to air. When the solution was saturated with O_2 and irradiated, decomposition was greatest.

TABLE 4.—Effect of N_2 and O_2 on the decomposition of benzo[*a*]pyrene by ultraviolet irradiation

Saturated with	Irradiation (hr)	Decomposition (%)
N_2	24	−8
	48	−16
O_2	24	−22
	48	−64
	114	−100

CONCLUSIONS

These experiments clearly showed that commercial fluorescent lamps of either daylight type or green-light type decompose some polycyclic aromatic hydrocarbons. Fluorescent lamps generally emit a fairly strong light at wavelengths less than 400 mμ. This fact should be considered whenever aromatic hydrocarbons are handled under fluorescent lamps. The sunlight also can cause serious destruction of the hydrocarbons and, even when weakened by passage through a glass plate, it decomposes some hydrocarbons in glass containers. It should be emphasized that prolonged exposure to these artificial and natural illuminations may cause serious destruction of the hydrocarbons; only transient exposure may not. When aromatic hydrocarbons are exposed to a strong ultraviolet light generated by a mercury vapor lamp, precautions should be taken. Presence of oxygen affects the photochemical degradation of aromatic hydrocarbons a great deal. Simple saturation of their solutions with N_2 does not guarantee satisfactory protection against degradation.

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(*2*) Cook, J. W., and Martin, R. H.: Polycyclic aromatic hydrocarbons. XXIV. J. Chem. Soc. 1125–1127, 1940.

(*3*) Leighton, W. G., and Forbes, G. S.: Precision actinometry with uranyl oxalate. J. Am. Chem. Soc. 52: 3139–3152, 1930.

DISCUSSION

DR. HOFFMANN: The original study by Wynder and Wright was done with dichloromethane as an eluting solvent. Later it was found that this solvent even in the dark caused quantitative changes in benzo[*a*]pyrene and other polynuclear aromatic hydrocarbon concentrations, despite the fact that it was highly purified. The other point we observed was the photo-oxidation of pure polynuclear aromatic hydrocarbons. We have to purify our polynuclear aromatic hydrocarbons every 2 weeks to be assured that our experiments can be repeated.

In our analytical laboratories we covered our lamps with a yellow plastic called "butyrate tenite," which absorbs all light below 470 mμ. Then we exposed solutions of hydrocarbons in cyclohexene to the light in this room. We could not find any changes in the ultraviolet spectra after a day of this treatment.

DR. COMMINS: I am very interested to hear of your results. We have studied the effect of sunlight on solutions of benzo[*a*]pyrene. I would like to ask you what amounts of hydrocarbons you had in your solutions, because we found that strong solutions of benzo[*a*]pyrene, for example, remain reasonably stable. They become slightly yellow, but the concentration remains more or less constant. A weak solution decomposes very rapidly. I would emphasize that standards for spectrophotometric analysis should be prepared immediately prior to use because the effect of sunlight on the solutions is quite marked. Just the light in the laboratory is enough to decompose the weak solutions of pure hydrocarbons. However, airborne particulate extracts are much more stable.

DR. KURATSUNE: I used a benzo[*a*]pyrene solution containing about 5 μg per ml.

DR. LYONS: I think the reason that strong solutions do not decompose as quickly as weak ones is that in the strong solutions the first few layers of molecules absorb most of the light energy. When you were irradiating your solutions, did you agitate them?

DR. KURATSUNE: No, I did not agitate them.

CHAIRMAN SAWICKI: I think another factor in the decomposition of a polycyclic hydrocarbon in solution would be the relative proportion of dissolved oxygen in the solution. Other things being equal, the more oxygen in solution, the more extensive the decomposition.

DR. ALTSHULLER: In the atmosphere itself would this photo-oxidation be of any importance? In other words, I wonder if there would be enough polynuclear material on the actual surface of the particle to be of any importance. Because here, of course, you would not have the added protection of your window glass. You would have the complete solar radiation and also, in oxygen-type smog such as in Los Angeles, you would have a secondary reaction possibly of the polynuclears with the ozone or atomic oxygen produced from the pyrolysis of NO_2. Would you please comment on that?

CHAIRMAN SAWICKI: Dr. Commins, would you like to comment on that?

DR. COMMINS: I really do not know an awful lot about oxides of nitrogen, but NO_2 itself certainly does decompose benzo[*a*]pyrene. If you expose a filter paper coated with benzo[*a*]pyrene to nitrogen dioxide, it goes yellow immediately. But I would like to comment later, when I will talk about the relative stability of samples in some detail.

DR. FALK: I tried to answer some of your points yesterday, when I showed the slide in which the exposure of the aromatics to smog and light caused some disappearance. In this study we did not differentiate between disappearance due to oxidation and that due to sublimation. All the smaller compounds obviously seemed to sublime easily, while the ones that disappear at a faster rate than the others might have been oxidized. The smog helps in this. I am sure that in our atmosphere nitrobenzo[*a*]pyrene could be made.

A Geometrical Aspect of Fluorescent Spectroscopy [1]

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SUMMARY

Fluorescent spectroscopy is a valuable tool for both qualitative and quantitative measurements of individual fluorescent components in polluted atmospheres. Unfortunately it is very difficult to interpret results because variations in the optics and geometry of the measuring system result in large variations in fluorescent spectra. Many of these variations and their causes are discussed. A general expression has been developed that relates fluorescent energy to incident energy over a wide range of concentrations. The expression covers the Beer-Lambert law region through the concentration quenching region.—NCI Monogr. No. 9: 127–133, 1962.

FLUORESCENT SPECTROPHOTOMETRY is frequently employed as a means of identifying arenes found in polluted atmospheres, but considerable care must be used as it is possible to obtain an infinite number of spectral variations from a single sample. The variations are due primarily to differences in the optics and geometry of the measuring system.

In an attempt to normalize fluorescent intensity measurements, an expression has been developed that allows the calculation of intensities at each wavelength for different concentrations. The expression utilizes a double application of Beer's law and is compatible with other similar developmental work (*1–6*). The expression, which is developed for a completely collimated system in which fluorescence is measured at right angles to the incident light, relates the intensity of fluorescent light at a single wavelength, the intensity of incident light at a single wavelength, the concentration, the thickness of the absorbing layer traversed by the incident light, and the thickness of the absorbing layer traversed by the fluorescent radiations.

INSTRUMENTATION

A high-pressure Xenon lamp coupled to a monochromator was used to obtain a relatively intense, stable, collimated, monochromatic, incident light. The fluorescent sample holder allowed adjustments for slit width and slit positioning. Fluorescent emissions were collimated at right angles and scanned through a second monochromator. Energy intensities as a function of wavelength were automatically recorded on an arbitrary scale.

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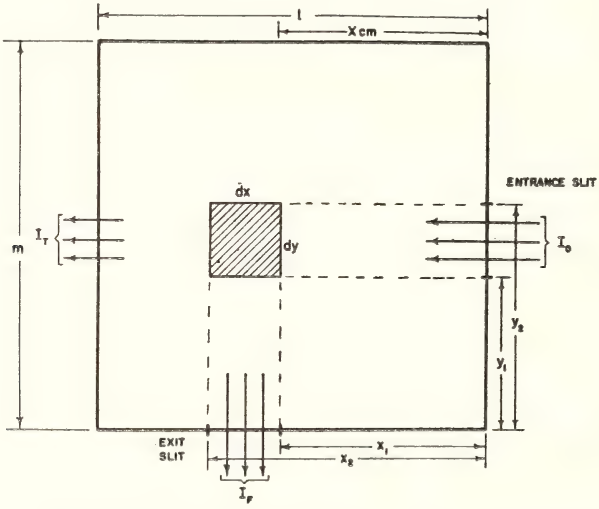
[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

THEORY

Text-figure 1 is an enlarged top view of a fluorescent sample cuvette with slit arrangement. The incident light of intensity, I_0, is passed through the entrance slit of width dy. The fluorescent light of intensity, I_F, is measured at right angles to the incident light and the transmitted light, I_T. The amount of incident light absorbed, I_A, in increment dx after passing through x cm of a solution at a molar concentration c is determined from a consideration of Beer's law:

$$I_{A\lambda_0(dxdy)} = I_{0\lambda_0} e^{-k_0 c x} dy - I_{0\lambda_0} e^{-k_0 c(x+dx)} dy.$$

Absorption coefficients, expressed as liters per mole cm, are represented by k_0, k_1, k_2 . . . corresponding to wavelengths of indicated interest, λ_0, λ_1, λ_2 . . . .



TEXT-FIGURE 1.—Top view of sample cuvette showing coordinate positions of entrance and exit slits.

By simplifying the expression and applying limits to x, as defined by the exit slit, it is possible to calculate the absorbed energy ($I_{A\lambda_0}$) contained within any length lamina of width dy in line with the incident light:

$$I_{A\lambda_0(x_1-x_2,dy)} = \int_{x_1}^{x_2} I_{0\lambda_0} k_0 c e^{-k_0 cx} dxdy. \quad (1)$$

The limits are measured in centimeters and are indicated in text-figure 1 by the symbols x_1 and x_2.

Compounds that fluoresce may convert all or part of the absorbed energy to fluorescent energy. The degree of conversion of the absorbed energy to the fluorescent energy contained within the solution (I_{0F}) and associated with a particular wavelength may be obtained by applying a conversion constant P_{λ_1}, P_{λ_2}, P_{λ_3}, . . .

$$I_{0F\lambda_1}=P_{\lambda_1}I_{A\lambda_0}. \tag{2}$$

In all expressions λ_1, λ_2, λ_3, . . . are wavelengths in the fluorescent region, generally associated with wavelengths of maximum fluorescent intensity; λ_0 is used to indicate the activation wavelength.

The constants P_{λ_1}, etc., may be compared to the conversion factor P which is used to relate total absorbed energy to total fluorescent energy· The degree of total conversion is referred to as the quantum yield of fluorescent efficiency.

The lamina or layer of fluorescent energy contained within the solution has a finite width. The width of this layer is defined by the entrance slit and corresponds to the limits imposed on the implied y of equation 1. These dimensions are measured in cm and are represented in text-figure 1 by y_1 and y_2.

A portion of the fluorescent energy contained within the solution is absorbed as it passes out through the solution. From a second consideration of Beer's law and application of the aforementioned limits to y, it is possible to determine the intensity of the emitted or transmitted fluorescent energy (I_F):

$$I_{F\lambda_1}=\int_{\lambda_1}^{\lambda_2} I_0F\lambda_1e^{-k_1cy}dy. \tag{3}$$

A general expression for calculating the intensity of fluorescent light at any wavelength emitted at a right angle to the incident light may be obtained by combining equations 1, 2, and 3:

$$\begin{aligned} I_{F\lambda_1}&=\int_{y_1}^{y_2}\int_{x_1}^{x_2} I_{0\lambda_0}P_{\lambda_1}k_0ce^{-k_0cx}e^{-k_1cy}dxdy \\ &=\frac{I_{0\lambda_0}P_{\lambda_1}}{k_1c}\cdot e^{-k_0cx}\Big|_{x_2}^{x_1}\cdot e^{-k_1cy}\Big|_{y_2}^{y_1}. \end{aligned} \tag{4}$$

For the expression to be universal it must include some unit height for I_F. The height used in all measurements presented herein was approximately 1 cm. This is a reasonable unit because it is less than the limiting height, which is the effective vertical dimension of the phototube.

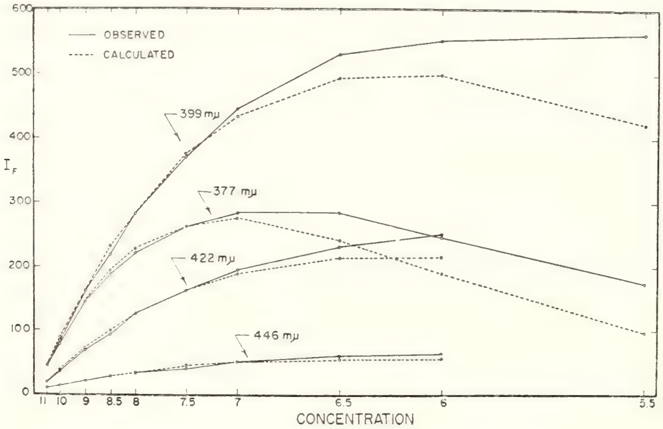
A simplified form of equation 4 may be obtained at very low concentrations.

$$I_{F\lambda_1}=I_{0\lambda_0}P_{\lambda_1}k_0c(x_2-x_1)(y_2-y_1) \tag{5}$$

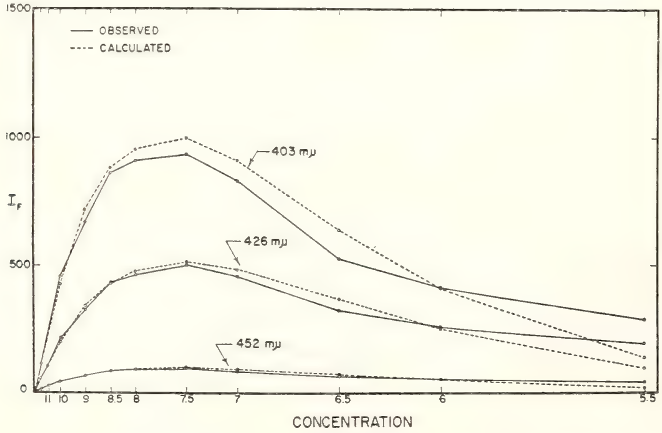
$$I_{F\lambda_1}\propto c.$$

RESULTS AND DISCUSSION

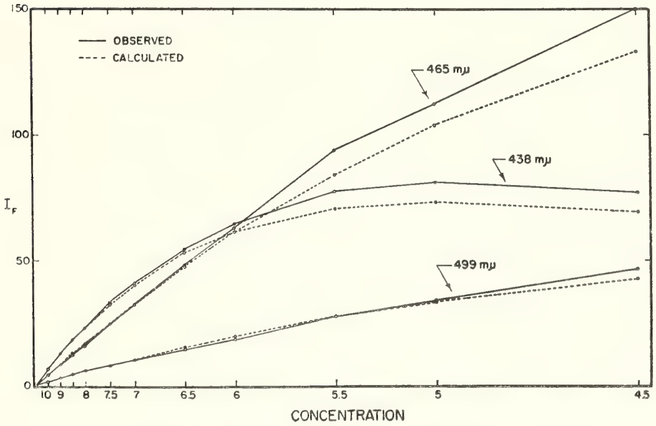
Graphical comparisons of calculated and observed values of fluorescent intensities are made in text-figures 2, 3, and 4 for anthracene, benzo[*a*]pyrene, and perylene in cyclohexane. The fluorescent intensities are presented on arbitrary scales, but may be normalized if presented in ratio form with the incident energy which is comparable to the conventional presentation of Beer's law. The concentrations are all based on an initial molar concentration, C_0, set at a value slightly below the saturation values for each compound. Serial dilutions by halves constitute all other concentrations, *i.e.*, $C_1 = \frac{1}{2} C_0$; $C_2 = \frac{1}{2} C_1$, etc. The values of C_{10} for each compound are presented in table 1 together with all other constants used in the calculations.



Text-figure 2.—A comparison of calculated and observed values of the fluorescent intensity of anthracene at various concentrations.



Text-figure 3.—A comparison of calculated and observed values of the fluorescent intensity of benzo[*a*]pyrene at various concentrations.



Text-figure 4.—A comparison of calculated and observed values of the fluorescent intensity of perylene at various concentrations.

Table 1.—Numerical values of all constants and parameters used in calculations of fluorescent intensities

C_{10} (γ/cc)	x_1 (cm)	x_2 (cm)	y_1 (cm)	y_2 (cm)	λ (mμ)	k	PI_0
				Anthracene			
1.109	0.48	0.52	0.48	0.52	357*	2.236×10^4	
					377	1.952×10^4	4.23×10^5
					399	Negligible	4.23×10^5
					422	Negligible	1.84×10^5
					446	Negligible	0.484×10^5
				Benzo[*a*]pyrene			
1.569	0.475	0.525	0.475	0.525	363*	5.45×10^4	
					403	5.45×10^4	60,500
					426	65.9	27,600
					452	18.3	5,230
				Perylene			
0.191	0.1	0.3	0.0	1.0	409*	6.68×10^4	
					438	7.61×10^4	6.8×10^2
					465	Negligible	4.4×10^2
					499	Negligible	1.4×10^2

*Activation wavelength.

As indicated in table 1, a combined value of PI_0 is given because of the difficulty in accurately measuring I_0. This difficulty is inherent in all spectrophotometers and is generally resolved, as mentioned, by use of a ratio presentation. The validity of the proposed expression can be checked without uniquely measuring P and I_0.

As indicated in text-figures 2, 3, and 4, agreement is very good between calculated and observed fluorescent intensity values for the 3 compounds tested. There is a small divergence at the higher concentrations because the same values for the extinction coefficients, k, were used throughout. Actually, at higher concentrations Beer's law is no longer obeyed and the uncertainty in k values makes all calculations doubtful in this concentration range.

The very close agreement between calculated and observed values at low concentrations indicates the validity of the straight-line plot of concentrations versus fluorescent intensities which is widely used in quantitative fluorescent measurements and is indicated in equation 5.

The color change that occasionally may be observed on diluting fluorescent solutions in the Beer's law region may be graphically exemplified in text-figure 4. The peak at 465 mμ is primarily responsible for the color associated with higher concentrations of perylene. As the concentration is decreased through the value corresponding to C_6, the peak at 438 mμ becomes most predominant with a resulting color change. A possible theoretical explanation is indicated by a consideration of the proposed expression; applying the unique absorption coefficients as well as the unique values of P associated with the wavelength of each peak allows a calculation of the exact concentration at which there is a reversal in the relative intensities of adjacent peaks.

The proposed expression may also be used as a basis to explain theoretically the phenomenon known as "concentration quenching." As the concentration is increased through a critical value, the fluorescent intensity of individual peaks, as well as the total fluorescent intensity, reaches a maximum and then decreases; text-figure 3 graphically exemplifies this. There is, thus, a strong indication that "concentration quenching" may be associated with absorption of the contained fluorescent light as it passes out through the solution.

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DISCUSSION

MR. BEGEMAN: I missed the comment you made on the solvent you used in your system.

DR. THOMAS: We used cyclohexane throughout. The solvent does not enter into the equation, but there would probably be different values of "P" and, of course, "K." The comparison is identical with the conventional treatment, as we have used Beer's law in this particular expression.

DR. FALK: Would you like to comment on the picture that you showed where you had not changed concentration, but got different maxima, different heights of the maxima just on the basis of geometric changes in position?

DR. THOMAS: In the position indicated in "A," we have most of the incident light absorbed before it reaches this point. A small amount of light that is fluorescent light within the solution is further absorbed as it passes out, which explains the very small curve that we obtained as indicated under "A."

Under "B," the incident light does not pass through a large value of "X," so we have a large amount of fluorescent light at this point. The fluorescent light then passes out, and there is a certain amount of absorption that occurs of that fluorescent spectrum. Then we have the curve as indicated at "B."

Now, "C." If you wanted to get the greatest sensitivity out of a spectrophotometer design, you would arrange your slits in this position where the incident light passes through the smallest amount of solution. The fluorescent light contained within then passes out through the smallest amount of solution. We have a curve corresponding to "C," and this is the highest curve. You will notice that we have a reversal here. With the values for "P" and "K," "K-1" at this point and "P" and "K-1" at this point, the calculations show the reversal as indicated in your developed expression. Then we have expression "D" where most of the incident light is absorbed. There is a small amount of fluorescence, but it has to pass out through just a small amount of solution, so we have this curve which is a little higher, curve "D" higher than curve "A." (A, B, C, and D appeared on slide used for oral presentation.)

DR. FALK: Concerning the figure you showed us of benzo[*a*]pyrene versus concentration ranges, you did not have that overlap in band heights.

DR. THOMAS: That was for a single program on the spectrophotometer.

DR. FALK: You had all the 3 maxima with unusual relative heights.

DR. THOMAS: Yes, but you see, I kept the fixed-slit arrangement. In this particular case there are 4 different programs on the curve that I compared. There was one program for benzo[*a*]pyrene. It was a face-centered slit. That work, incidentally, was done at the California State Department of Public Health with their Aminco Bowman spectrophotometer. The anthracene curve was on the Aminco-Keirs phosphorimeter, which I think is what you have in your laboratory. Again you have the face-centered slit arrangement. Your slits are measured on the Keirs in millimeters, where theirs are still indicated in inches. I used the smallest possible slit arrangement so that I could get adequate sensitivity and enough resolution. The slit was 0.05 cm. Actually that is the lowest you can get. The final figure was one where I used the makeshift spectrophotometer. The entrance slit is centered on the entire face of the cell, and the exit slit is 0.2 mm off-center.

We have tried other arrangements, and the equation works out as well as indicated on the schematic drawing.

DR. TIPSON: When you press the button to let light into the instrument and take a fluorescence reading immediately, then repeat again and again, do you always get the same reading or do you get a diminished one every time you press the button and take the reading?

DR. THOMAS: Some compounds are more sensitive to light than others. In this particular case we tried to take different compounds and concentrations. We assumed that there was no change in concentration even though we knew that some change was occurring. These compounds were slightly affected by the light. This is a slow process. There is not an instrument on the market that would give us absolute readings. The Aminco does give you a scan of the fluorescence spectrum.

Separation and Identification of Aliphatic and Aromatic Carcinogens From Environmental Sources[1, 2]

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SUMMARY

Methods for separation of carcinogenic aromatic hydrocarbons, heterocyclics, and their derivatives from environmental sources by column and paper chromatography are discussed. The identification of these compounds by paper chromatography and spectroscopy is described. Recent developments concerning the effects of solvents and *p*H on fluorescence and the use of these methods as diagnostic tools in the examination of carcinogens from environmental sources also are described. This series is exemplified by compounds of the carbazole and acridine series. Some oxy compounds are known carcinogens; the possibility of their presence in air and the methods of analysis were examined.—NCI Monogr. No. 9: 135–151, 1962.

OUR WORK on the chemistry of cigarette smoke has been reported in the literature (*1, 2*). However, I would like to make a few brief comments concerning our methods of separation and identification. Cigarette smoke is a very complex mixture of hundreds of organic compounds, and column chromatographic procedures are undoubtedly not as effective with it as they are with less complex mixtures such as air pollutants. Column chromatography did not give fractions that were clean enough for ultraviolet identification. Text-figure 1 shows the ultraviolet spectrum of a column chromatographic fraction with alumina as the adsorbent—before and after paper chromatography.

We used paper chromatography followed by ultraviolet and fluorescence spectroscopy for the identification of the compounds in cigarette smoke. Some typical paper chromatograms are shown in figure 1. These are all column chromatographic fractions from cigarette-smoke condensate. The banded separations were used for the treatment of larger quantities of material. All these separations were obtained with Tarbell's procedure

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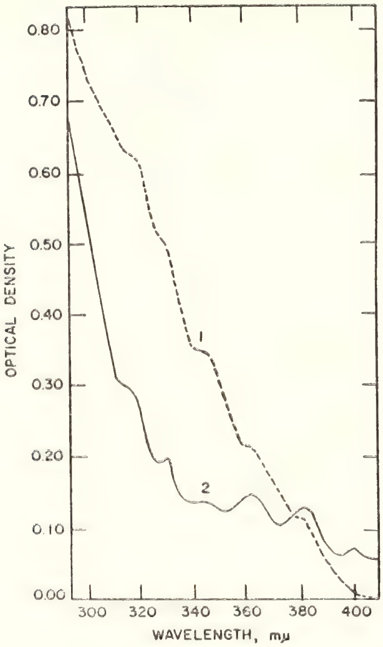
[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

[2] This work was supported by the American Cancer Society, and instrumentation was purchased through research grants CS-9571, and CS-9713 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] The author is indebted to Dr. Norton Nelson for his advice and encouragement. Biological tests in our laboratory were carried out under the direction of Dr. Norton Nelson and Dr. Leo Orris.



TEXT-FIGURE 1.—Ultraviolet spectrum of a column chromatographic fraction with alumina as the adsorbent—before and after paper chromatography.

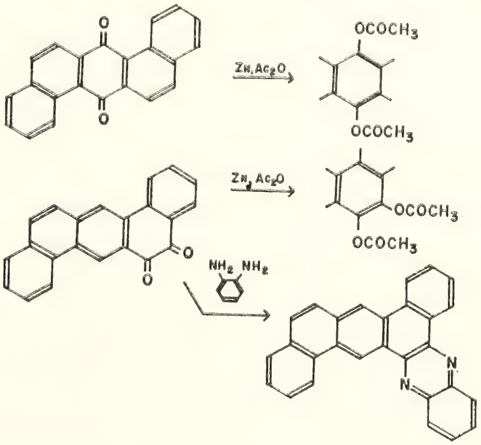
(*3*), in which dimethylformamide-hexane was used as the solvent with Whatman #1 paper. Other workers have used different methods with equal success, *e.g.*, the methanol-ether-water (4 : 4 : 1) system, developed by Wieland and co-workers (*4*), with acetate paper, and Bergmann's (*5*) toluene-methanol-water system, also with acetate paper. It is often advantageous to use 2 chromatographic systems in succession. In table 1, the R_F values of the 2 dibenzcarbazoles are the same by the first method, but are different by the second method, which uses acetate paper. The 2 systems were used successively for complete separation of the dibenzcarbazoles and dibenzacridines from cigarette-smoke condensate (*1*). The question is often asked as to which paper chromatographic system is best. This is difficult to answer since we were primarily interested in showing the presence of a number of carcinogenic hydrocarbons and heterocyclics in cigarette smoke. With our aims accomplished, the relative merits of the various systems were not further explored. We have obtained recoveries of the order of 95 percent or better from spots on which 1μg or less of material was chromatographed. With larger quantities of material, considerable losses due to trailing occurred. We have established the quantities of material that give the best recovery values with our paper chromatographic systems and hydrocarbons.

The chemical and analytical procedures we used in the cigarette-smoke analyses were extended recently in a more fundamental study, namely, that of the metabolism of aromatic carcinogens (*6*). Since these metabolic products are all derived by oxidation of the carcinogen, they

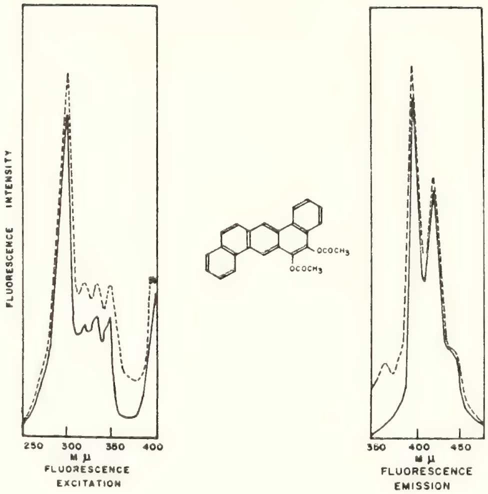
TABLE 1.—R_F Values of nitrogen heterocyclics

Compound	R_F Values Whatman #1 paper (*N,N*-dimethylformamide-hexane)	 Acetate paper (toluene-methanol-water; 1:10:2)
7*H*-Dibenzo[*a,g*]carbazole	0. 15	0. 18
7*H*-Dibenzo[*c,g*]carbazole	0. 15	0. 30
Dibenz[*a,h*]acridine	0. 61	0. 12
Dibenz[*a,j*]acridine	0. 35	0. 35

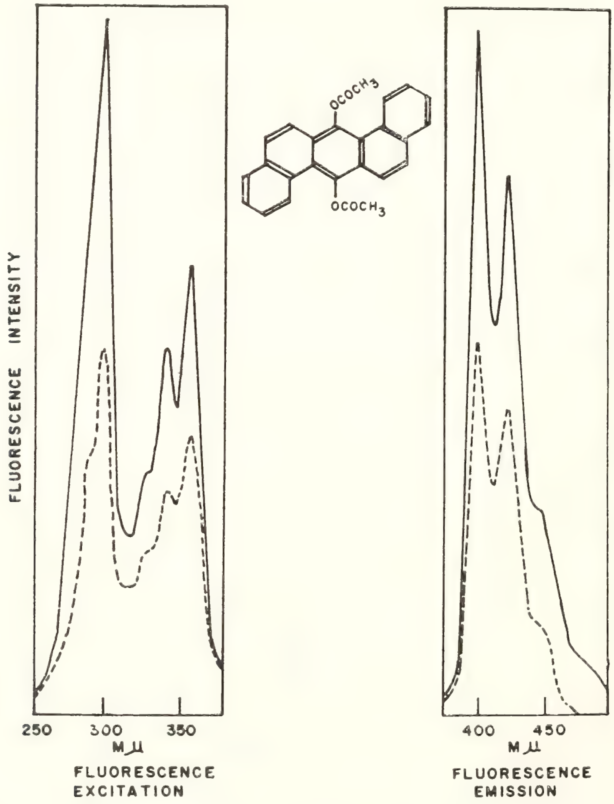
may be of concern to those in air pollution research who are interested in finding and analyzing such oxidation products of aromatic hydrocarbons. The metabolites we studied were obtained from the rat lung metabolism of dibenz[*a,h*]anthracene. Microgram and submicrogram levels of these products were obtained and their identification presented a new challenge. Prominent among these products were the 5,6- and 7,14-quinones of dibenz[*a,h*]anthracene (text-fig. 2). These quinones do not ordinarily fluoresce in organic solvents and remain at the origin of paper chromatograms with most of our systems. Their ultraviolet spectra show broad bands that are not readily distinguished from background absorption. A well-known chemical reaction, reductive acetylation, with zinc dust and acetic anhydride was used for conversion of this mixture of quinones from the metabolism of dibenz[*a,h*]anthracene to their respective hydroxy-acetates, as shown in text-figure 2. Also, the ortho-quinone readily forms a strongly fluorescent quinoxaline with *o*-phenylenediamine. All these derivatives were readily paper-chromatographed. The R_F values and, as expected, the fluorescence excitation and emission spectra differed for the 5,6-diacetate and the 7,14-diacetate, as shown in text-figures 3 and 4, respectively. Another metabolite, the dihydrodiol, gave distinct spectra (text-fig. 5). The ultraviolet and fluorescence spectra of this compound, however, are very similar to those



TEXT-FIGURE 2.—5,6- and 7,14-quinones of dibenz[*a,h*]anthracene.

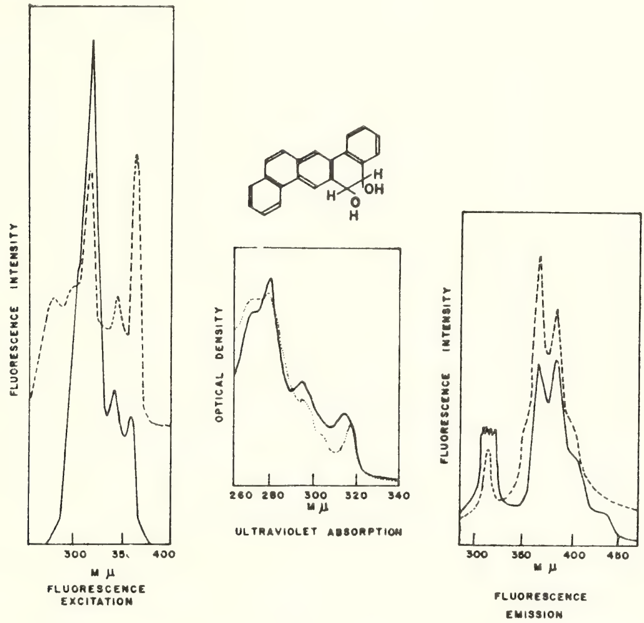


Text-figure 3.—Fluorescence excitation and emission spectra for 5,6-diacetate. Pure compound (- - -); compound from mixture (—).

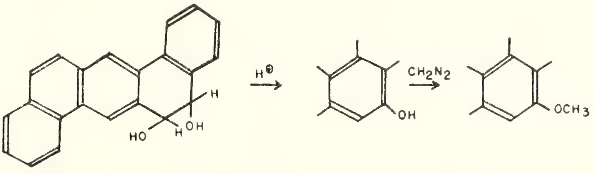


Text-figure 4.—Fluorescence excitation and emission spectra for 7,14-diacetate. Pure compound (- - -); compound from mixture (—).

of other compounds with the same phenylphenanthrene moiety. Its presence was confirmed by the reaction sequence shown in text-figure 6, *i.e.*, dehydration and conversion of dihydrodiol to the ether with diazomethane. The methyl ethers of these phenols are separated much more readily than the parent phenols by the same system of paper chromatography used for the hydrocarbon.

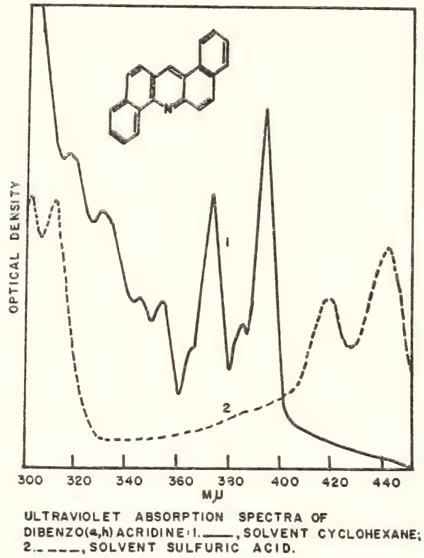


TEXT-FIGURE 5.—Fluorescence excitation, emission, and ultraviolet spectra for dihydrodiol. Pure compound (—); compound from mixture (- - -).

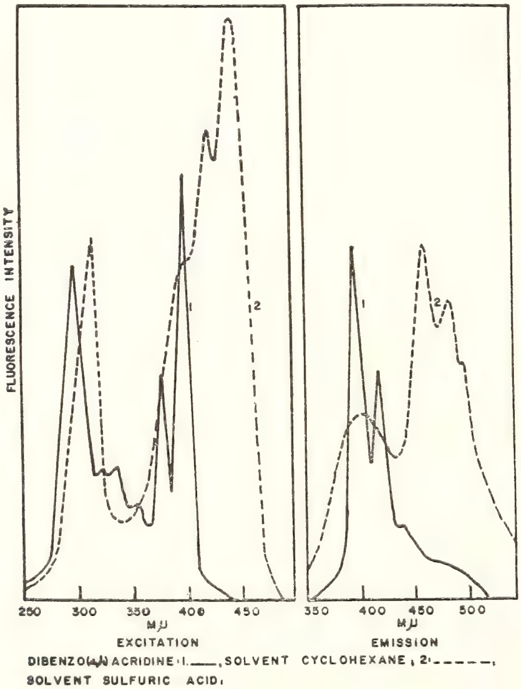


TEXT-FIGURE 6.—Dehydration and conversion of dihydrodiol to ether with diazomethane.

The use of fluorescence as an analytical tool in the identification of aromatic hydrocarbons and heterocyclics has expanded considerably in the past few years. Some exploratory work along these lines has been carried out in our laboratory, where we studied the effects of solvents on the fluorescence of a group of heterocyclic compounds. These compounds are not carcinogens, but our results are of interest since they point to the potentialities of the examination of such solvent effects for analytical and assay purposes. In fact, some work along these lines already is reported in the literature. Text-figure 7 shows the ultraviolet absorption spectra of dibenz[*a,h*]acridine (a carinogenic heterocycle that we isolated from cigarette tars) in cyclohexane and in sulfuric acid. A shift to longer



TEXT-FIGURE 7.—Ultraviolet absorption spectra of dibenz[*a,h*]acridine in cyclohexane and in sulfuric acid.

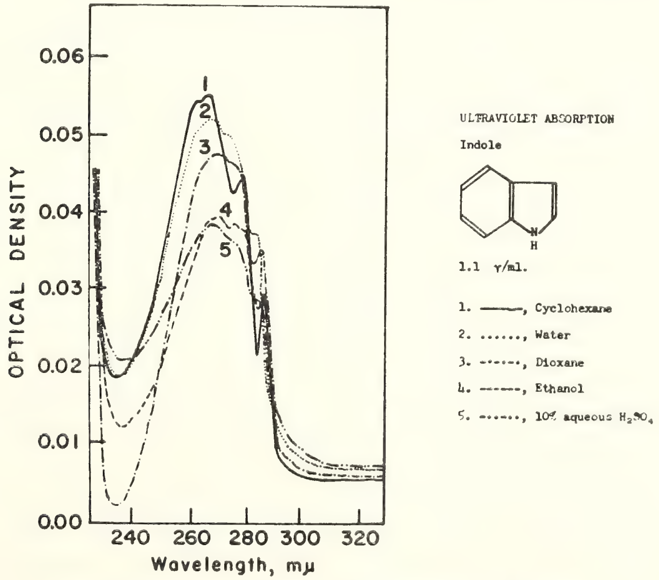


TEXT-FIGURE 8.—Fluorescence excitation and emission spectra of dibenz[*a,h*]acridine in cyclohexane and in sulfuric acid.

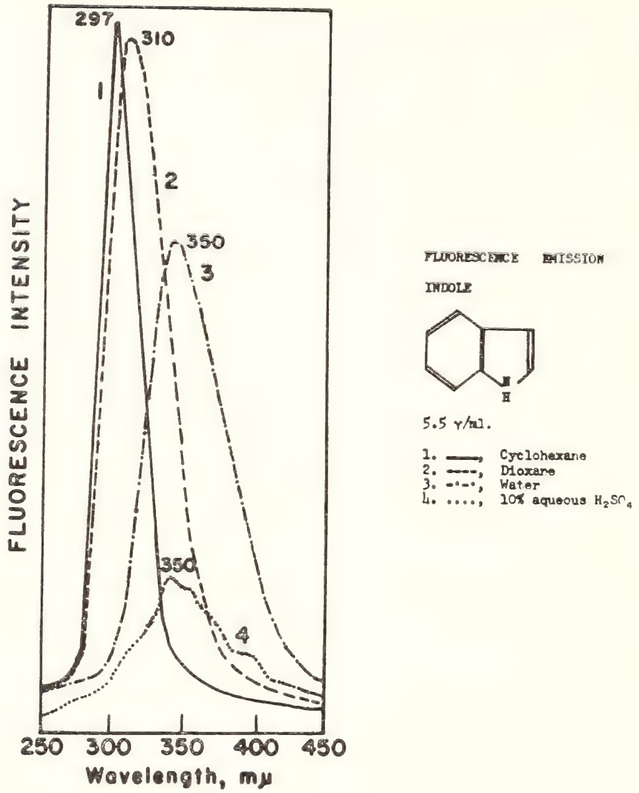
wavelength is indicated in sulfuric acid; this shift to longer wavelength is also reflected in the fluorescence excitation and emission spectra shown in text-figure 8. The shift is typical of the usual solvent effects reported in the literature. In some compounds, however, shifts occurring in the fluorescence emission spectra are not accompanied by comparable shifts in the ultraviolet absorption and in the fluorescence excitation spectra.

We have examined some of these compounds in detail. The ultraviolet absorption of indole in cyclohexane, water, dioxane, ethanol, and dilute aqueous sulfuric acid is shown in text-figure 9. It is apparent that change of solvent has little effect on the band positions, although the resolution and, to a minor extent, the intensities are affected. The fluorescence excitation maxima of indole in various solvents were identical also; however, large changes in the fluorescence emission spectra occur with a change of solvent. Text-figure 10 shows the emission of indole in cyclohexane, dioxane, water, and aqueous sulfuric acid. All these spectra were obtained at the same concentration, 5.50 μg per ml, and at the same excitation wavelength, 282 mμ. In going from cyclohexane to water, a shift of 53 mμ occurs. Text-figure 11 shows the effect of ethanol, and text-figure 12, those of trichloroacetic acid and benzene. Sharp decreases in fluorescence intensity occur in the presence of both trichloroacetic acid and in benzene. We have examined the theoretical implications of these results as applied to indole and its derivatives and have reported our findings in the literature (*7*). These findings are pertinent in this discussion only in that the solvent effects can be discussed in terms of solvent polarity and hydrogen bonding.

The fluorescence spectra of alkyl derivatives of aromatic compounds are usually very similar to those of their parent compounds. In the



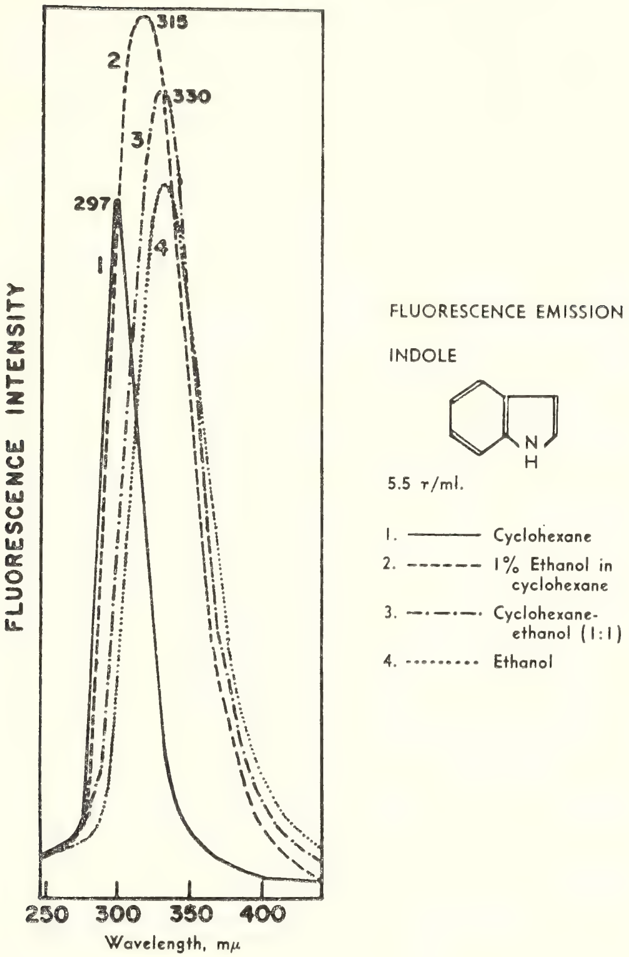
TEXT-FIGURE 9.—Ultraviolet absorption of indole.



Text-figure 10.—Fluorescence emission spectra of indole.

methylindoles shown in table 2, however, there are shifts of from 5 to 25 mμ from one compound to the next in the same solvent; *e.g.*, in water, indole shows a maximum at 350 mμ and 2,3-dimethylindole a maximum at 376 mμ. In dioxane solution, indole is readily distinguished from the various methylindoles, whereas in cyclohexane, 2- and 3-methylindole are readily distinguished. The ultraviolet spectra of all these methylindoles are very similar and are not affected by the solvent. The relative fluorescence intensities of some of the methylindoles are markedly decreased in water and in benzene; in fact, those of the 2- and 3-methylindoles in benzene were so weak that the 5 mμ slits ordinarily used were replaced by 10 mμ slits to provide more energy. Fluorescence data on 1,2-dimethylindole and 1-methyl-2-phenylindole are given in table 3. In going from cyclohexane to water, both these compounds show shifts to longer wavelengths, but their ultraviolet spectra remain unchanged. 1-Methyl-2-phenylindole in various solvents exhibits a much higher fluorescence intensity than the other compounds in this series.

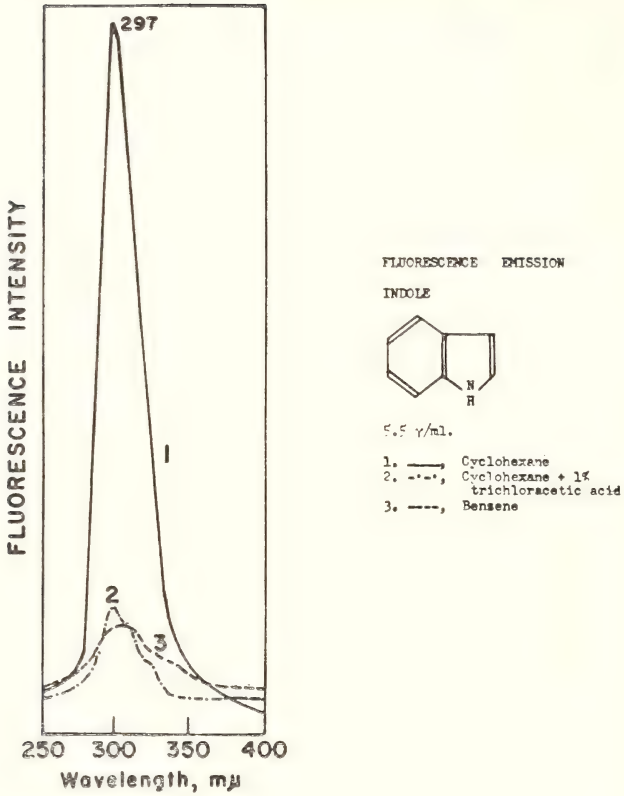
Unlike the other indole derivatives, 3-acetylindole does not fluoresce in any of the solvents. Its ultraviolet absorption data (table 4) show shifts to longer wavelength on going from a solvent with a low dielectric constant to a solvent with a higher dielectric constant.



TEXT-FIGURE 11.—Fluorescence emission spectra of indole.

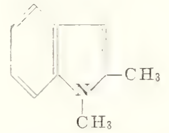
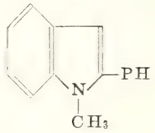
TABLE 2.—Fluorescence of indole and methylindole

Solvent	Emission maxima (mμ)			
	Indole	2-Methylindole	3-Methylindole	2,3-Dimethylindole
Cyclohexane	297	306	315	320
Benzene	305	305	317	330
Dioxane	310	325	329	340
Ethanol	330	335	350	360
Water	350	355	370	376
Excitation wavelengths	282–285	270–280	275–285	280–285



Text-figure 12.—Fluorescence emission spectra of indole.

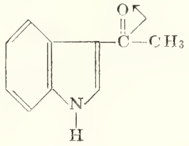
Table 3

Solvent	 1,2-Dimethylindole			 1-Methyl-2-phenylindole		
	Fluorescence			Fluorescence		
	Excitation (mμ)	Emission (mμ)	R.F.I.*	Excitation (mμ)	Emission (mμ)	R.F.I.*
Cyclohexane	291	315	10. 0	310	360	64. 0
Benzene	291	326	11. 0	310	370	63. 0
Dioxane	291	329	7. 7	310	370	72. 0
Ethanol	291	340	11. 0	310	370	61. 0
Water	291	362	7. 9	305	380	17. 0

*Microammeter readings at same sensitivity setting, same slit width, 5 mμ, and same concentration, 4.7 × 10^{-5} moles per liter.

Indole-3-acetic acid does not fluoresce measurably in cyclohexane or benzene, but it does fluoresce in dioxane, ethanol, and water, with shifts to longer wavelengths in that order (table 5). The fluorescence intensities

TABLE 4



3-Acetylindole
Fluorescence: None
UV Absorption:

Solvent	λ max (mμ)	ε max	λ max (mμ)	ε max	λ max (mμ)	ε max
Cyclohexane	233	7, 250	249	(shoulder)	280	5, 800
Dioxane	237	9, 400	252	6, 380	288	11, 020
Ethanol	240	12, 850	257	9, 300	293	12, 620
Water	241	7, 970	257	8, 250	296	11, 740

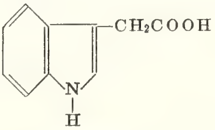
in these solvents are comparable to those of the other indole derivatives, and the ultraviolet spectra remain essentially unchanged with change of solvent.

Fluorescence emission maxima of carbazole shift to longer wavelength in solvents with greater dielectric constants; however, the shifts are smaller than those for the indoles and, in addition, comparable shifts are observed in the ultraviolet absorption spectra with changes of solvents.

The pyridine series was examined next. Pyridine itself, of course, does not fluoresce, and pure quinoline fluoresces only weakly; the next higher analogue, acridine, does fluoresce (*8*). We examined the fluorescence of acridine and its angular isomers, 5,6-benzoquinoline, 3,4-benzoquinoline, and 7,8-benzoquinoline. The fluorescence of acridine in various solvents is shown in table 6. The fluorescence maxima move to longer wavelengths, and fluorescence intensity increases in solvents with greater dielectric constants. Furthermore, the fluorescence intensity is remarkably weak in cyclohexane and in benzene, which necessitates greater light intensities for observation of this fluorescence. In acid, however, the fluorescence intensity of acridine is increased vastly; in addition, there is a pronounced shift to longer wavelength. The strongest peak in the excitation spectrum of acridine in all solvents and in acid is at 355 mμ. The ultraviolet absorp-

TABLE 5

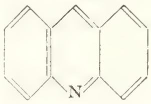
Indole-3-acetic acid



Solvent	Fluorescence		R.F.I.*
	Excitation (mμ)	Emission (mμ)	
Cyclohexane	No fluorescence		
Benzene	No fluorescence		
Dioxane	290	325	8. 5
Ethanol	290	340	6. 3
Water	290	360	5. 3

*Microammeter readings at same sensitivity setting, same slit width, 5 mμ, and same concentration, 4.7 × 10^{-5} moles per liter.

TABLE 6.—Fluorescence of acridine*



Solvent	Fluorescence emission maximum	Fluorescence intensity
Cyclohexane	400	Very weak †
Ethanol	400, 420, 445	1. 0
Water	430, 455, 475	8. 2
10 Percent H_2SO_4	457, 484, 505, 515	30. 0

*One μg per ml; 5 mμ slits; excitation at 355 mμ.
†Twenty mμ slits.

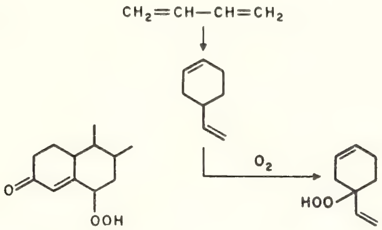
tion spectra in the first 3 solvents are essentially identical. In acid the 355 mμ peak is increased in value.

In contrast, the 3,4-, 5,6-, and 7,8-benzoquinolines, *i.e.*, the angular isomers of acridine, do not show any solvent effects. Acridine, therefore, occupies an unusual position in this series, and this behavior can be used to distinguish it from its angular isomers.

An extension of these studies to other aromatics, particularly those in the heterocyclic series, should yield useful information for qualitative and quantitative analyses of aromatic air pollutants.

The results of our fluorescence analyses of mixtures of hydrocarbons (*8*) are of direct concern in studies of separation and identification of carcinogens. A mixture containing 1 μg each of anthracene, pyrene, fluoranthene, dibenzo[*cd,jk*]pyrene, benzo[*a*]pyrene, and benzo[*ghi*]fluoranthene was prepared. Emission spectra of this mixture were obtained at excitation wavelengths of from 250 to 400 mμ at 10 mμ intervals. All the emission spectra from 250 to 300 mμ and from 320 to 390 mμ were identical and agreed with the fluorescence emission spectrum of benzo[*a*]pyrene. The spectra obtained with excitation at 310 and 400 mμ agreed with the emission spectrum of dibenzo[*cd,jk*]pyrene. The other 4 compounds could not be detected in the presence of the strongly fluorescent benzo[*a*]pyrene. Since there are several satisfactory methods for the paper chromatography of aromatic compounds, no further attempts were made to analyze complex mixtures directly by fluorescence spectroscopy.

The fourth phase of our work consisted of an examination of carcinogenic oxy compounds, which included the carcinogenic hydroperoxides, peroxides, epoxides, and lactones, on which only sporadic carcinogenicity tests were carried out. Kotin (*9*) has postulated that epoxides and peroxy compounds formed by the oxidation of olefins are responsible, or partly responsible, for the carcinogenicity of polluted air and has induced pulmonary tumors in mice with ozonized gasoline. Epoxides, hydroperoxides, and peroxides are, of course, particularly suspect since it is conceivable that they will be formed by autoxidation or photochemical oxidation of olefins in the air. These compounds are of interest to us primarily because a study of their carcinogenicity could yield information about the early stages in the metabolism of aromatic carcinogens. Thus, it has been postulated that epoxides are intermediates in the formation of dihydrodiols (*10*). If this is true, such epoxide intermediates would be of interest since carcinogenicity has been reported (*11*) for diepoxybutane and vinylcyclohexenedioxide. Fieser (*12*) has reported carcinogenicity for a sterol hydroperoxide, the partial structure of which is shown in text-figure 13. We prepared the hydroperoxide of butadiene dimer and found that, when painted on the skin of mice, this compound was indeed carcinogenic.



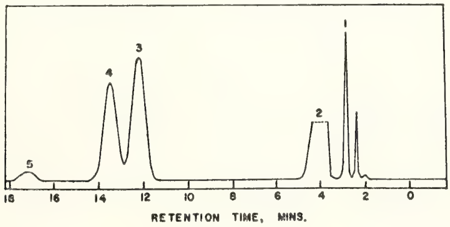
Text-figure 13.—Partial structure of a sterol hydroperoxide and the formation of 1-vinyl-1-hydroperoxy-cyclohexene-3.

Not all epoxides, of course, are carcinogenic; various epoxides that we tested, which included oleic and linoleic acid epoxides and squalenehexaepoxide, are not carcinogenic.

Currently, we are preparing and testing a series of the latter compounds for carcinogenic activity. Once the structural features required to confer carcinogenicity in these compounds are identified, air pollution researchers will be able to examine their products for these materials.

We have found vapor phase chromatography and infrared spectroscopy effective in the analysis of these compounds from reaction products. With the aid of vapor phase chromatography, for example, we were able to study the epoxidation of 3-epoxy-butene-l with peracetic acid (text-fig. 14). The 2 isomers of diepoxybutane, which differ only stereochemically, were readily separated by this method, and preparative vapor phase chromatography provided samples for infrared spectroscopy.

In summary, I have described briefly some areas of our research on chemical carcinogenesis, which may be of interest to air pollution special-



Text-figure 14.—Epoxidation of 3-epoxy-butene-1: 1,3-epoxy-butene-1; 2, chloroform; 3, meso-1,2,3,4-diepoxybutane; 4, *d*,1-1,2,3,4-diepoxybutane; 5, unidentified by-product of reaction.

ists. These include the identification of aromatic hydrocarbons and heterocyclics from cigarette smoke; the identification and structure determination of oxidation products of dibenz[*a*,*h*]anthracene; some aspects of fluorescence spectroscopy; and finally, the significance of non-aromatic oxy compounds as possible air polluting carcinogens.

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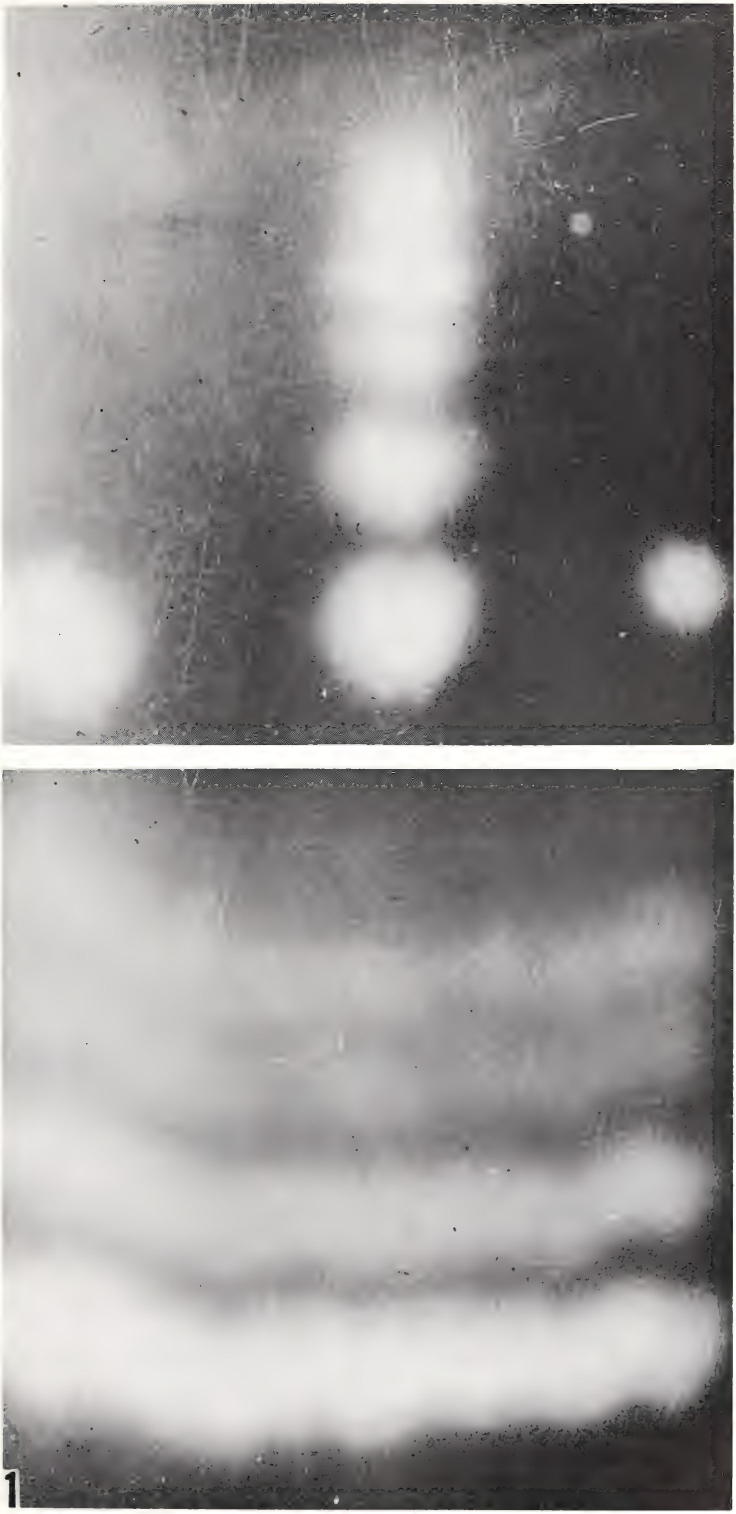
Plate 13

Figure 1.—Typical paper chromatograms from cigarette-smoke condensate.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DISCUSSION

DR. HOFFMANN: Dr. Van Duuren, which concentration did you use for your vinylcyclohexenehydroperoxide when you applied it to mouse skin?

DR. VAN DUUREN: It was one-half of 1 percent in benzene.

DR. HOFFMANN: Thank you very much. Dr. Van Duuren, can the fluorescence studies of indole derivatives be used in the study of tryptophane metabolism? This would be an enormous tool.

DR. VAN DUUREN: Yes, I hope that this kind of work will be of value to people who are interested in that area of research. I know that very much of it is going on at the National Institutes of Health.

DR. HOFFMANN: Dr. Van Duuren, you isolate the dihyroxydihydrodibenz[*a,h*]anthracene and you identified it by reaction and finally as a methoxy derivative. Do you think this methoxy derivative might be present as one metabolism product of dibenz[*a,h*]anthracene since Schoental and Berenblum have shown that 8-methoxybenzo[*a*]pyrene has a high carcinogenic activity? I heard recently a paper given by Dr. Berenblum, and he thinks that this strong carcinogen might be a metabolism product.

DR. VAN DUUREN: From the point of view of mechanism of formation I cannot see any reason why it should be formed metabolically. I do not believe that any of these types of ethers were ever isolated from the metabolism of an aromatic hydrocarbon. Of course, these compounds do occur as conjugates, glucuronides, sulfates, and phosphates. Dr. Falk may have a comment on that.

DR. FALK: I read that paper and obviously looked for the 3-hydroxy compound. First of all, in the bile of a rat, not a trace of 3-hydroxybenzo[*a*]pyrene was found. This compound is formed later by hydrolysis of the glucuronide conjugate. I found no trace of the methoxy derivative, even though I looked for it high and low.

DR. BADGER: I wonder if I may ask whether the configuration of the diol of dibenzanthracene was determined. The cis-diol is, of course, obtainable synthetically from the hydrocarbons plus osmium tetroxide.

DR. VAN DUUREN: There is one point that I did not stress enough, and that is that we have been working with microgram and submicrogram levels of material. It would be interesting to know whether it is a cis or the trans compound. As soon as we can get enough material, we will put it in the infrared machine. That certainly would tell us.

DR. FALK: Did you use monoepoxides for your carcinogenic testing or only diepoxides?

DR. VAN DUUREN: We tested the epoxides of linoleic and oleic acids and squalenehexaepoxide.

DR. FALK: And did both groups produce tumors?

DR. VAN DUUREN: None of them did. But there is reported carcinogenicity for the diepoxy butanes and for vinylcyclohexene dioxide.

DR. FALK: Yes, we produced tumors with diepoxides and with monoepoxides when we tested them by injection in mice.

Some Problems Concerning Analysis of Polycyclic Hydrocarbons in Particulate Pollution Samples [1]

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SUMMARY

Methods for the analysis of polycyclic hydrocarbons in particulate pollution samples were reviewed. Because of the very low concentration levels of these substances, extreme accuracy in analysis was required. A standard approach to analysis should be evolved to allow useful comparison of data on potential carcinogens in varied communities and countries. A principal problem was the loss of polycyclic hydrocarbons during sampling and analysis. Oven-drying and prolonged storage were undesirable. Loss of the substances by volatilization was also a problem. Ultraviolet degradation was illustrated by the results obtained on a sample of chrysene isolated from Ottawa air. Other adverse effects considered were the uniformly high background in the ultraviolet spectra of air pollution samples, the use of unsuitable analytical techniques, confusion in identification standards, and possible mistakes in the identification of compounds of closely related structure. Identification by means of fluorescence measurements at optimum excitation wavelengths was considered; the method requires extensive confirmatory work. The need for high purity of the standards used as reference compounds was emphasized.—NCI Monogr. No. 9: 153–169, 1962.

SINCE THE earlier work of the British, Wedgwood and Cooper (*1*), Cooper (*2*), Commins (*3*), Lyons and Johnston (*4*), and others (*5*), the instrumentation available for the identification of potential carcinogens in the atmosphere has been improved greatly. With refinement of techniques and elaboration of methods, nevertheless, the number of carcinogens isolated from air samples, vehicular exhausts, etc., has not increased significantly, and the complexity of the subject has become more apparent. The urgent requirement in further work, it is becoming increasingly evident, is a redefinition of objectives and a closer coordination between bioassays and analytical programs, rather than the development of involved analytical routines. The problem of resolving and identifying all of the two dozen or more polynuclear hydrocarbons reported as detectable in air samples is complex and intriguing analytically, but justification for the time and effort involved is questionable. The practical requirement is a simple, well-defined and standardized procedure which will permit quantitative comparisons of the carcinogenic potentials of the atmospheres of various localities, classified with respect to types and quantities of fuels consumed, climatology, etc. If the primary objective of work on atmospheric carcinogens is thus defined, methods of collecting samples, questions of chemical stability during analytical manipulation, the practical requirements of those responsible for the assessment of biological activity of airborne hydrocarbons or groups of hydrocarbons, and questions regarding the atmospheric reactions which modify or eliminate the airborne polynuclears, all require investigation.

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[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

For Liverpool, England, Commins (*6*) has reported an average dust loading over a 6-month interval of more than 900 μg per m^3 of air. In the United States and Canada an average particulate loading of about 200 μg per m^3 would be considered high. To collect comparable weights of solids, greater volumes of air must be sampled in the United States or Canada than in the United Kingdom. Also, the combustion of coal for domestic heating is a relatively greater source of pollution in the United Kingdom and Europe than in North America. These two factors can be assumed to change the basic patterns of the problem in North America and in Europe. For example, common experience shows that the volatility of naphthalene, anthracene, and other members of the polycyclic group is substantial at temperatures far below their melting points. It is likely, therefore, that the aspiration of large volumes of air through fiber glass sheets, necessary on this continent to obtain a significant weight of particulates, results in low and indeterminate collection efficiencies for the various hydrocarbons and that comparisons of concentration data are subject to a high degree of qualification.

In Canada, air pollution problems are in general less acute than in the United Kingdom and United States. The very large concentrations of population, such as in London, New York, and Los Angeles, are not a feature of the country. Steam locomotives have been entirely eliminated from the railways, and coal as a domestic fuel has been to a large extent replaced by fuel oil and gas. Nevertheless, heavy concentrations of industry, increasing traffic density in moderate-sized cities, local topographical and climatological features, and other variables result in problems which are closely comparable with those in the United States and United Kingdom. With regard specifically to atmospheric carcinogens, staff limitations have permitted only a relatively modest effort in Canada. Shore and Katz (*7*), Moore and Katz (*8*), and Dubois *et al.* (*9*) have reported on some 8 or more polycylics found in urban atmospheres, and concentrations of pyrene, fluoranthene, benzo[*a*]pyrene, and coronene have been estimated. A number of larger Canadian cities are cooperating in the collection of particulate samples in a manner suited to the determination of polynuclears, and advanced instrumentation will permit the investigation of recently formulated ideas on the subject.

Insofar as can be judged from the subjects announced for this symposium, it would seem that the time is opportune for an objective analysis of previous work in the field and for discussion of objectives, methods, and programs for future work. Before we proceed, therefore, to a detailed description of some of the problems encountered in our work, it is worthwhile to mention our recent experience with some of the newer techniques. Thin layer chromatography has been investigated to a point where it appears that it might be applicable to the analysis of the neutral aromatics and might be useful for preparative purposes. Vapor phase chromatography has been investigated somewhat more extensively. Problems in preparation and injection of the sample, choice of solid support and stationary phase in the column, and choice of detector have not been resolved. Phosphorimetry has not yet been investigated experimentally in this laboratory. We think that these, and other approaches, merit an exchange of ideas and the expression of speculative opinion.

The problems encountered in past work, which will be discussed in some detail, are summarized as follows:

1. Volatility of polynuclears.
2. Incomplete resolution on columns.
3. Degradation by exposure to ultraviolet radiation.
4. Difficulties in interpretation.
5. Shortcomings of sampling techniques.
6. Solvent effects in fluorescence.
7. The ambiguity of fluorescent intensity measurements.
8. Nonreproducibility of R_F values in paper chromatography.
9. Contamination and irregularity of acetylated paper.
10. Unsatisfactory purity of reference standards.

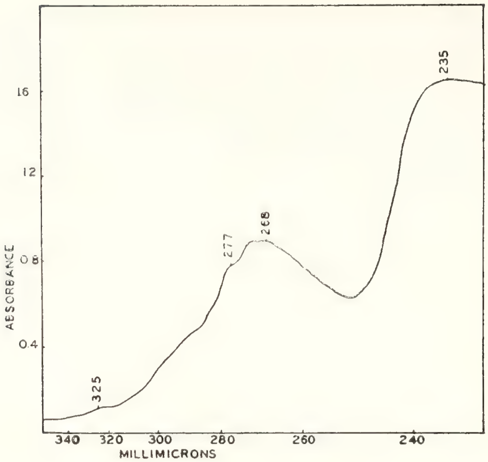
SPECIFIC PROBLEMS

Volatility of Polynuclears

The ultraviolet absorption spectrum of a 3 ml isooctane extract of a diesel exhaust sample is illustrated in text-figure 1. The exhaust particulates collected during the idling phase of the operating cycle of a 1,750 horsepower diesel were brownish and greasy, and benzene extraction showed a low insoluble content. The glass fiber sheets used for collection were extracted with benzene within 2 weeks of sampling. No heat was used to dry the samples. A conventional fractionation was carried out on a silica gel column into aliphatic, aromatic, and oxygenated fractions. Qualitatively, the absorption peaks illustrated seem to indicate naphthalene or alkyl naphthalenes.

Three points in this curve are worth noting: 1) Naphthalene in an undried diesel exhaust sample leads to the surmise that (since diesel exhausts are a constituent of the general air pollution load) even brief oven-drying of air particulate samples changes the quality of the extractables significantly.

2) The finding of naphthalene in the supposedly aliphatic fraction indicates that fractionation on a silica gel column is not exact with respect to the resolution of aliphatic, aromatic, and oxygenated groups. We had previously come to this conclusion, when with a sufficient volume



Text-figure 1.—Ultraviolet absorption spectrum of diesel exhaust.

of isooctane, it was possible to elute pyrene from air samples. The indicated course of future work is either to refine the method of group separation or to do away with it.

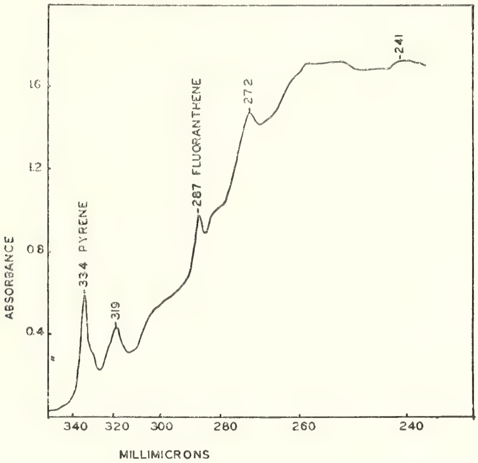
3) The identification of the material whose spectrum is shown as naphthalene is obviously open to question. Supplementary fluorimetric curves helped to identify the material as probably naphthalene or a naphthalene derivative, but neither ultraviolet absorption, or fluorescence, or both together could exactly identify the substance.

The importance of naphthalene as a constituent of the polynuclears is, perhaps, trivial. It does serve to point out that losses of the more volatile polycyclics can be lessened during sampling and subsequent treatment.

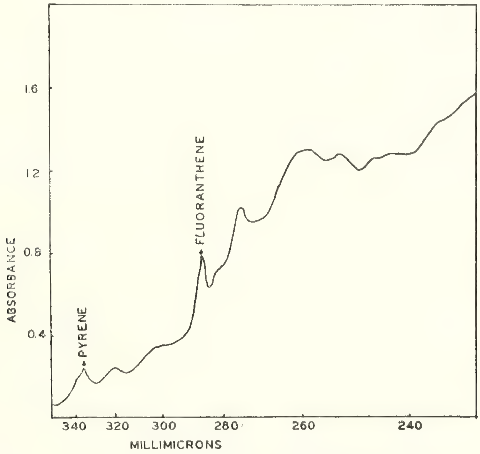
In this connection, Commins and Lawther (*10*) discuss benzo[*a*]pyrene losses due to volatility during sampling, and Dubois *et al.* (*9*) indicate the possibility of anthracene losses during paper chromatography. More generally, consideration of the analytical difficulties in such a relatively simple case emphasizes the troubles to be expected with the more complex polynuclear hydrocarbons. With naphthalene, it seems reasonable to expect that the parent substance might be associated with varying quantities of alkyl derivatives, hydro derivatives, and incipiently oxygenated products. Some, or all, of these constituents are sufficient to shift the diagnostic ultraviolet absorption peaks a few millimicrons, or enough to make positive identification doubtful. In our limited experience with the fluorimetry of air samples, similar remarks would seem to apply to fluorescence spectra.

Incomplete Resolution

The ultraviolet absorption spectrum of a composite of cyclohexane fractions from a column is shown in text-figure 2. Pyrene structure predominates. Measurement of the 334 mμ peak indicated 29 μg of pyrene. The peak at 286 mμ represents about 17 μg of fluoranthene. Text-figure 3 is the spectrum of a composite of elution fractions showing decreasing amounts of fluoranthene. The respective quantities of pyrene and fluoranthene were 4.5 and 13.0 μg.



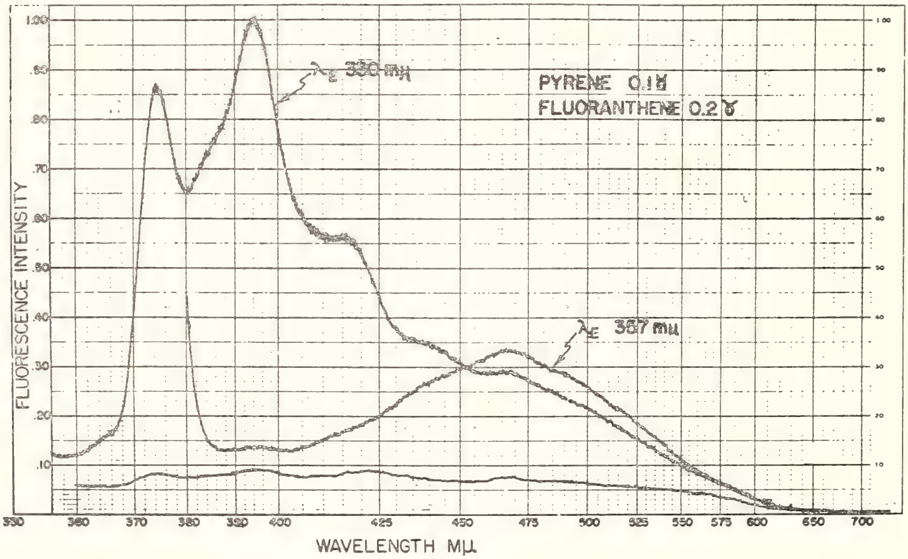
Text-figure 2.—Ultraviolet absorption spectrum of a composite of cyclohexane fractions from a column.



Text-figure 3.—Spectrum of a composite of elution fractions showing decreasing amounts of fluoranthene.

These two curves illustrate the difficulty of resolving pyrene and fluoranthene on a column. It is relatively easy to select small elution fractions, as column chromatography progresses, which show predominant structure for either pyrene or fluoranthene with only trace indications of one or the other. When quantitative estimation is required, however, the limit of practicability, we believe, is about as shown. The quantities mentioned were derived from about 9000 m^3 of Ottawa air sampled in the middle of March, 1961. The estimated dust loading was about 68 μg per m^3. On this basis, the total pyrene concentration was 0.37 μg per 100 m^3 and that of fluoranthene was 0.33 μg per 100 m^3.

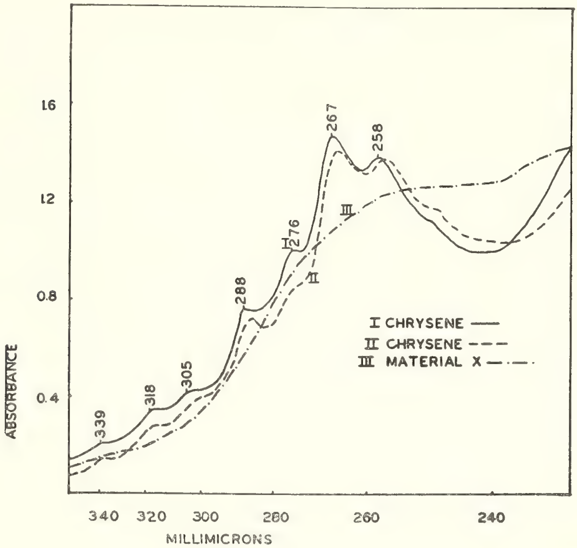
Considerable difficulty has been indicated in the liquid chromatographic analysis of pyrene and fluoranthene. It must also be mentioned that these isomers do not separate any better by paper chromatography. The best method of distinguishing or determining pyrene and fluoranthene in liquid or paper chromatograms is probably fluorescence spectroscopy of the two-component mixture. At suitable actuation wavelengths the individual fluorescence spectra can be obtained. This is illustrated in text-figure 4 (*11*).



TEXT-FIGURE 4.—Fluorescence spectra of pyrene and fluoranthene.

Degradation by Ultraviolet Light

Text-figure 5 shows the spectrum of a composite sample representing predominantly chrysene in curve I. The sample represented by curve I was then taken up on alumina and added to a silica gel/cyclohexane column. Elution was carried out under continuous exposure to ultraviolet radiation. The ultraviolet spectrum of the eluate removable from the column with cyclohexane plus benzene is shown in curve II. The spectrum was determined in isooctane and shows a slight shifting of peaks but essentially the same structure as curve I. Curve III is the spectrum in isooctane of a fraction removable from the column with alcohol. This material gave a sharply defined brown-yellow zone only partly soluble in isooctane. The results suggest the usefulness of a quantitative repetition of the work with a carefully purified sample of



TEXT-FIGURE 5.—Ultraviolet spectrum of a composite sample representing predominantly chrysene.

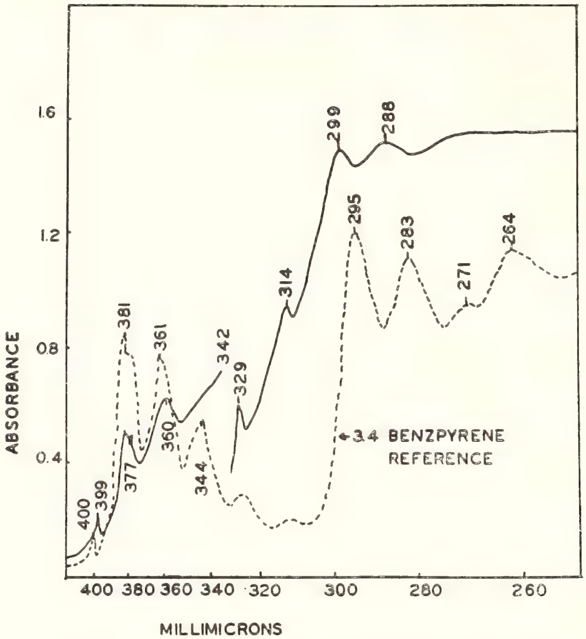
chrysene in quantity sufficient to permit investigation of the brown-yellow background material.

The preceding results are in agreement with Miller and Baumann (*12*), who found that a group of 6 polynuclears, including benzo[*a*]pyrene, were destroyed by ultraviolet radiation. (Miller and Baumann did not work with chrysene.) In contrast, they found that dibenzanthracene was not destroyed by ultraviolet light. Obviously, care must be taken to avoid undue exposure of polynuclears to ultraviolet light during sampling, extraction, and measurement.

Difficulties in Interpretation

A spectrum in isooctane of a fraction which might best be described as probably containing benzo[*a*]pyrene is shown in text-figure 6. It was prepared from a composite of fractions which, on the original column, were only vaguely suggestive of benzo[*a*]pyrene structure, by rechromatographing on somewhat less active alumina than that of the original column and then on a second column of active silica gel.

The sequence of manipulations necessary to prepare a spectrum which is still less than definitive is, in our opinion, too involved to be practical. Our main purpose in showing the curve is to demonstrate the difficulty of getting even ambiguous results with small concentrations of benzo[*a*]pyrene. It would be desirable, in our opinion, that papers reporting concentrations of benzo[*a*]pyrene should show reproductions of the actual spectra from which the reported concentrations are calculated. The foregoing is an example of the troubles encountered in the analysis of air samples by methods which are described as simple, rapid, and accurate.



Text-figure 6.—Spectrum of an isooctane fraction probably containing benzo[*a*]-pyrene.

We believe that such methods, based on the analytical isolation of the components of synthetic mixtures of pure constituents or tests assumed to be specific in the presence of interferences, leave much to be desired. The question of the so-called background material in unknowns has not been dealt with in an entirely satisfactory way. This material appears, from ultraviolet absorption curves, to be present in greater quantity than the materials looked for. Until the nature of the background material is known with more certainty, the exact analysis of air samples by ultraviolet absorption is seriously handicapped. Our impression from preliminary experience with fluorescence is that fluorometric methods may add a degree of certainty to the interpretation of ultraviolet absorption data, but that either or both are not completely definitive.

Simplification of Sampling and Analysis

In the foregoing, we have indicated that some of the current sampling and testing methods may not be satisfactory. We believe that too much emphasis is placed on samples taken on glass fiber sheets. This is convenient, but it is probable that sample efficiency is not the same for all polynuclears. The loading effect and the unevenness of sample deposition are well known.

The adverse effects of heat in the drying of glass fiber samples to constant weight has been mentioned. In the past, conventional treatment of the sample has involved exposure to heat, air, and ultraviolet light. This has resulted in numerous air sample analyses in which the predominant polynuclear was coronene, with little evidence of anything else. This is probably due to the lower volatility and greater stability of coronene as compared with others of the group.

Sampling in a liquid absorbent is advantageous because subsequent extraction is eliminated and the over-all process is speeded up. It is simpler and more direct and should be free from the adsorption effects associated with carbonaceous material caught on the glass fiber at the same time.

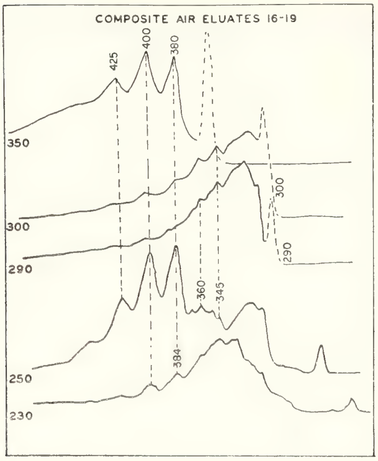
In this connection, Balgairies and Claeys (*13*) suggest sampling by aspirating air through purified spectrograde cyclohexane and measuring the fluorescence at a selected wavelength. The value obtained is called the anthracene index I_A and is a representation of the fluorescent intensity of 100 ml of cyclohexane through which has been aspirated 100 liters of air. It is reported that 0.0001 μg of anthracene per ml gives a measurable fluorescence. Sawicki *et al.* (*14*) report a relative fluorescent intensity, K_Q value, of 7.4 at 350 mμ for anthracene compared with a K_Q value of 23.1 at 380 mμ for benzo[*a*]pyrene. Balgairies and Claeys use the 3865 A mercury line for excitation. With the stronger excitation and continuous energy now available in the new Xenon arc lamps and with the flexibility of some of the new instruments, much improvement apparently may be made in sensitivity and specificity.

Preliminary experiments with this sampling technique were made on consecutive air samples aspirated in cyclohexane at the same source. Fluorescence spectra were determined directly as the sample was removed from the bubbler. It was evident from the spectra obtained that the air sample had a distinctive pattern which was constant for the duration of sampling. Column chromatography of the cyclohexane samples resulted in the fluorescence curves shown in text-figures 7 and 8. The preliminary spectra were obtained minutes after receipt of the sample; the separation necessary to produce the other curves required only 2 hours. One of the predominant hydrocarbons would seem to be anthracene. In text-figure 9 are shown excitation and emission spectra of a composite column eluate of the air sample in which anthracene structure is indicated unambiguously in the emission spectrum.

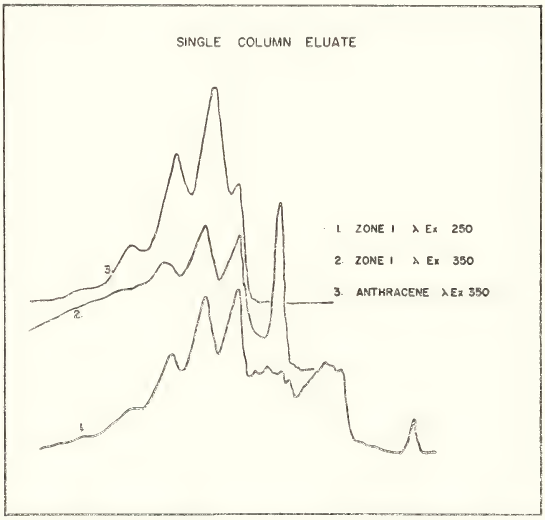
Solvent Effects on Fluorescence

In quantitative work there are intensity losses due to concentration quenching, localization of fluorescence, and self absorption. These effects may be decreased by making the measurements on sufficiently dilute solutions. As a practical criterion, Chaudet and Kaye (*15*) adjust the ultraviolet absorbance of the solution to 0.015 per cm, measured at 365 mμ, which results in concentrations of about 1×10^{-6}. The concentrations suggested by Sawicki (*14*) are of the order of 1×10^{-5}.

According to Miller and Baumann (*16*), fluorescence is inhibited by dissolved oxygen. They found that the fluorescence intensities of hydrocar-

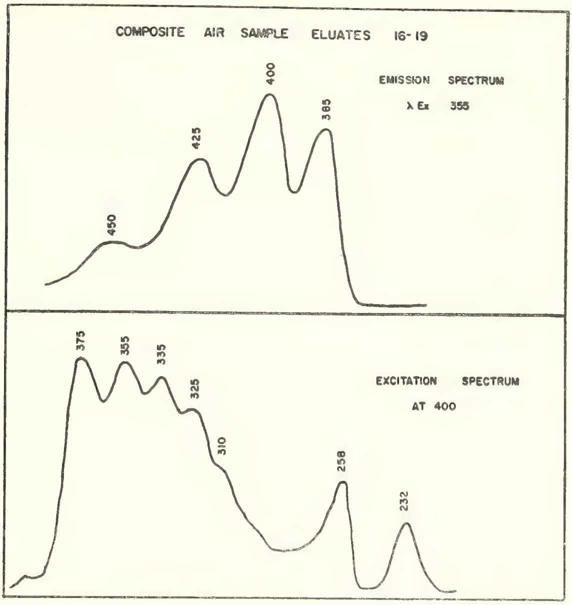


Text-figure 7.—Fluorescence curves from column chromatography of cyclohexane samples.



Text-figure 8.—Excitation and emission spectra of a composite column eluate.

bons such as benzo[*a*]pyrene varied hyperbolically with the partial pressure of oxygen. It is possible to remove oxygen from the solution with a stream of nitrogen before measurement. Also, the sample may be evacuated to remove dissolved oxygen. The solvent used has its effect, the fluorescence of benzo[*a*]pyrene in ethyl ether being double the fluorescence in petroleum ether, for the same concentration of benzo[*a*]pyrene and oxygen. Tetrahydrofurfuryl alcohol, pyridine, and dioxane dissolve comparatively small amounts of oxygen and show large fluorescence intensities in the presence of air. These three solvents, incidentally, contain ether-type linkages. Nitrobenzene dissolves little oxygen, but completely inhibits the fluorescence of the hydrocarbons. According to Jones (*17*),



TEXT-FIGURE 9.—Excitation and emission spectra of air-sample eluate.

nitration of benz[*a*]anthracene to the mono-nitro derivative results in complete loss of fluorescence. Sulfur dioxide is a much more potent inhibitor of fluorescence than oxygen, and the fluorescence of benzo[*a*]pyrene is completely and reversibly inhibited in concentrations of 10 μg per ml when the solvent is saturated with SO_2 at 740 mμ.

The choice of the best solvent for fluorescence work may have to be a compromise. It should be free from fluorescence or easily made so. Preferably, it also should be transparent in the ultraviolet, to allow application of both ultraviolet absorption and fluorescence measurements. If possible, it should have a low affinity for oxygen, but a high solubility for polycyclic hydrocarbons. It should be stable, and neither react with nor inhibit its fluorescence.

Balgairies and Claeys *(13)* state that pyridine, though a desirable solvent, cannot be produced without fluorescence. Cyclohexane is not a particularly good solvent for polynuclear hydrocarbons. Chlorinated and nitro compounds are unsuitable. The former in the presence of ultraviolet radiation rapidly destroys the fluorescence of the hydrocarbon. This suggests that the use of methylene chloride as a preliminary extractant in the processing of glass fiber samples may be undesirable.

Relative Intensity of Fluorescence

As is well known, the intensity of fluorescence varies with the particular hydrocarbon. In the form of a chromatogram spot, 0.001 μg of benzo[*a*]pyrene is easily visible to the eye under ultraviolet illumination.

On the other hand, it is difficult or impossible to see microgram amounts of chrysene or phenanthrene on a paper chromatogram. The relative fluorescent intensities, K_Q, according to Sawicki (*14*) are benzo[*a*]pyrene 6.0, chrysene 0.6, and phenanthrene 0.2, which is consistent with the paper chromatographic behavior. This also may explain why Van Duuren could not find pyrene, fluoranthene, and anthracene present in equal amounts with benzo[*a*]pyrene (*18*). In this case the K_Q's in order are 0.5, 0.8, 3.0, and 6.0. The only compound detectable in this mixture was benzo[*a*]pyrene (*18*). We believe, however, that when enough data have been accumulated relative fluorescence intensities from paper chromatogram spots may show certain disagreement with those for solutions. For example, we find fluorene essentially nonfluorescent on paper. In Sawicki's tabulation (*14*), it is rated somewhat ahead of fluoranthene. Is the behavior of fluorene in solution and on paper so different, or is it possible that samples contain small amounts of a highly fluorescent impurity, thus accounting for the high K_Q? Considering fluoranthene, we would rate it much stronger than anthracene and perhaps more intense than benzo[*a*]pyrene. These discrepancies in order of intensity may be related to the fact that the colors of fluorescence for a given hydrocarbon may be different in the solid and liquid state (*19*).

Paper Chromatography

Up to the present time we have had difficulty in obtaining reproducible R_F values. With acetylated paper, reproducibility within the sheet, from sheet to sheet and from batch to batch, leaves much to be desired.

This variability is partly due to temperature variations in our present laboratory and partly, we believe, to inherent nonuniformity of the paper. Some improvement in R_F values obtained recently seems to be associated with the use of "test tube" chromatography, instead of the previously used large cabinets and cylinders.

Acetylated paper, as manufactured at present, is apparently acetylated after the sheet has been made. On examination in ultraviolet light each lot shows signs of rinse marks and gross contamination. The impurities mentioned fluoresce yellow or orange. It would appear that a block of paper sheets is acetylated without individual separation. This could account for the uneven acetylation and evidence of uneven rinsing. The over-all fluorescent background may be lowered by pre-extraction with suitable solvents or by making a prechromatographic blank solvent run. Both these precautions are undesirable and represent considerable extra work.

It is suggested that the paper should be acetylated and washed in the fiber stage. This should permit the preparation of a uniformly acetylated fiber. The paper sheets could then be prepared from the raw material. Improvement should result in the control of degree of acetylation, and uniformity and cleanliness of the finished sheets. It should then be reasonable to expect all sheets in a lot to be uniform in performance.

We have mentioned our hope of achieving some consistency in R_F measurement. Separations, though not perfect, are in a better position. With air samples the multitude of zones present and the tendency toward a streaking fluorescent background result in very complex and crowded chromatograms. With the instruments now available for identification, it is hoped that crowding can be reduced by the use of smaller sample aliquots. Paper chromatography remains, at present, a valuable separation technique. For example benzo[*a*]pyrene and benzo[*ghi*]perylene, considered difficult to separate (*14*), are easily separated on paper.

Ultraviolet absorption or fluorescence measurements applied to chromatogram spots suffer from high background and scatter. It is hoped that interference can be reduced by the use of new media with lower backgrounds and by appropriate treatment of the paper.

A new instrumental approach is suggested such as X-ray diffraction analysis of a paper chromatogram spot. We have under construction a photomultiplier photometer that we hope will permit total relative fluorescence intensity measurements on paper spots.

Gas Chromatography

Our experience with gas chromatography has not been too successful to date. Suitable samples and suitable instrumentation are a prerequisite. The sample must, for example, contain reasonable amounts of polynuclears, it must be possible to inject the sample and maintain it in vapor form, and the detector must possess adequate sensitivity.

We have experimented with the simpler aromatics, such as naphthalene, anthracene, 9-methylanthracene, phenanthrene, and diphenyl, with some success, using the solution-syringe-injection technique. However, anthracene and phenanthrene do not separate but appear as one peak.

Another approach was to build a heated adapter to plug into the gas sampling valve. Air samples, taken on a glass fiber sheet, were mounted on this device and efforts were made to take the chromatogram. With this technique we were less successful than with the simple injection technique. We believe that failure with these air samples was due to the fact that only very small amounts of polycyclics were present. When standard amounts of naphthalene were handled in this apparatus, recoveries were low, possibly because of local condensation in the rather involved apparatus.

We have had some success with air samples in simple solvents, using a cold trap condenser loop attached to the gas sampling valve, and hope that we may be able to apply this to polycyclic samples. It must be remembered that if the air sample is passed through material such as silica gel the peaks of interest may be "swamped" by the large quantity of water which is likely to be present. Two obvious expedients suggest themselves: 1) a predryer in the sampling train and 2) a dual function column, with the first section consisting of a water vapor absorbent.

In our previous work, we have used thermal conductivity detectors. These detectors, we realize, are not as sensitive as the ionization detectors that are now so popular, but they have the advantage of not indicating "false" positives. In this connection, it should be pointed out that the flame ionization detector does not respond to water vapor, a distinct advantage with certain air samples.

A few words might be said about instrumentation. Almost without exception, the instruments available are not well designed, particularly with regard to the injection and rapid vaporization of samples and the introduction of solid samples. Detector cells are likely to be hard to clean, and the connections usually have too many bends, making local condensation and loss possible. In one case, we improved the exit tubing from the detector of a commerically available instrument by eliminating 10 right-angle bends in a space of perhaps 7 horizontal inches. This resulted in a perfectly straight tube from the detector, of minimum length, which was easily heated and gave a very short time lag between the recorder and the fraction collector.

It is anticipated that gas chromatography will eventually become a useful method for polycyclics; some disadvantages may be repeated at this point. The method, with any detector, is essentially nonspecific. Downstream identification of fractions at the main detector exits by appropriate chemical or physical means is desirable, so that some specificity of response may be obtained. Thermal conductivity or β-ray ionization detectors do not destroy the sample, and therefore the above suggestion has some validity. Unfortunately, the sample is destroyed in a flame- or thermocouple-type detector. Specific identification, in the latter type, would be difficult and would have to take place before the sample reaches the detector.

The instrument makers at present do not seem to have the special requirements of air pollution analysis in mind. It is hoped that this may be changed, to permit the full application of this technique. Recently, as a matter of fact, several manufacturers have produced injection systems said to be suitable for solid samples. The high-sensitivity detectors introduce two problems. The carrier gas must be pure enough that foreign peaks will not be added to the chromatogram. Suitable stability, or conditioning, of the stationary phase is necessary to avoid the possible contribution of peaks from this source.

One of the most promising approaches in gas chromatography is the use of exceedingly low concentrations of a stationary phase in a glass bead support. This, according to Hishta *et al.* (*20*, *21*), results in the separation of some of the lower polycyclics at much lower temperatures than are usually required.

CONCLUSIONS

The foregoing discussion emphasizes some of the shortcomings and difficulties encountered in methodologies for the analysis of polycyclic hydrocarbons in particulate air pollution samples. The concentration levels of these substances in the community atmosphere, particularly in North America, are so low that in 100 m^3 of air they occur only in μg quantities or less, or in quantities of less than 5 to 20 parts per 100,000 by weight of particulates. Extreme accuracy in methods of identification and quantitative analysis is essential if a useful cross comparison of data on potential carcinogens in the air of various communities in different countries is to be achieved. This requires the evolution of a standardized approach to techniques of collection of particulate atmospheric impurities, *i.e.*, separation of the organic fraction by designated solvents followed by systematic identification of the important polynuclear hydrocarbons by ultraviolet and fluorescence methods. Perhaps a similar approach in which the major, potentially carcinogenic, fraction is isolated and determined quantitatively may be the most suitable method for standardization purposes. The determination of a carcinogenic "index" would be highly desirable in the characterization of community atmospheres.

Since many commercially available polycyclic hydrocarbons have been shown to be impure (Dubois *et al.*), it is important that this situation be improved. We have found it necessary to prepare some of our standards, *e.g.*, chrysene, by paper chromatography. This procedure, at present, cannot easily be avoided, but only microgram amounts of a pure standard can be prepared in this way. More work is required to raise the purity of the standards, since all the analytical techniques are based on a "pure" reference standard. In particular, it is desirable to interest the chemical supply houses in preparing such polycyclics to the higher standards of purity required.

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DISCUSSION

DR. VAN DUUREN: On behalf of those of us who have been very laboriously separating aromatic hydrocarbons from environmental sources, I would like to take strong exception to Dr. Katz's statement concerning the publication of spectra. There are in the literature, and have been for some years, some very excellent spectra from various laboratories.

The second point that I would like to comment on is on the separability of pyrene and fluoranthene. These two compounds can, indeed, be separated very nicely by paper chromatography. The impurity in chrysene which has been discussed was identified by Buu-Hoi as 5*H*-benzo[*b*]carbazole. This work was published last year (Mabille, P., and Buu-Hoi, N. P.: J. Org. Chem. 25: 1937, 1960).

CHAIRMAN SAWICKI: The presence of 5*H*-benzo[*b*]carbazole in chrysene can be quickly determined by dissolving the chrysene sample in alkaline dimethylformamide. A brilliant yellow fluorescent solution is obtained. This impurity could be readily alkylated in the alkaline solvent and in this way the chrysene could be purified. We also use this technique in characterlzing carbazole and other polynuclear carbazoles, some of which are carcinogenic. Very characteristic excitation and emission fluorescence spectra are obtained.

DR. HOFFMANN: I agree fully with Dr. Van Duuren about the publication of spectra. The reason we do not show the ultraviolet absorption spectra of various hydrocarbons is because they are readily available.

DR. BADGER: I would like to ask one question in connection with the acetylated paper. I was not quite sure whether Dr. Katz said that you might get a better paper if you acetylated the pulp and then made the paper or whether you buy a better paper.

DR. KATZ: We have not solved this problem of the acetylated paper to our satisfaction. We have suggested to the manufacturers that they should acetylate the pulp, and at least try, and get a more uniform acetylated paper than the present samples that are being turned out.

DR. BADGER: I would like to draw attention to the paper published by Beckwith and Thompson last year on the phenylation of phenanthrene, where they provided a test to check whether acetylated cellulose was good or not. This test involves a standard column. In the separation of perylene from benzo[*a*]pyrene under their conditions you should be able to get 7-inch separation.

Determination of Paraffin Waxes of Tobacco and Tobacco Smoke by Gas-Liquid Chromatography[1]

NICOLA CARUGNO, *Laboratorio Chimico dei Monopoli, Rome, Italy*

SUMMARY

Paraffinic hydrocarbons in tobacco and tobacco smoke were determined. Tobacco contains C_8 to C_{34} alkanes and tobacco smoke contains C_{15} to C_{33} alkanes. It is shown that high-temperature gas-liquid chromatography can be used in the search and determination of paraffins of tobacco and tobacco smoke. The complete analysis is rapid and eliminates any need for complete fraction action of paraffin waxes for determination of single paraffinic hydrocarbons.—NCI Monogr. No. 9: 171–181, 1962.

PARAFFINS IN tobacco leaf and tobacco smoke and their composition have been the object of investigations by several researchers, particularly in recent years, because of their importance as probable carcinogens or as precursors of polycyclic hydrocarbons formed during smoking (*1–10*).

Barbezat (*11*) investigated the complexity of composition of the paraffins extracted from tobacco and tobacco-smoke condensate. He determined that these include solid paraffins with 25 to 30 carbon atoms, some with low melting points and some that are liquid at standard temperature. Chromatography on vegetable charcoal does not permit isolation of a normal pure paraffin.

Kosak and Swinehart (*12*) found by mass spectroscopic analysis that isoalkanes are present in the paraffin-wax fraction of cigarette smoke. To extend research in this field, high-temperature gas-liquid chromatography was used for analysis of the paraffins in tobacco and tobacco smoke. This method had already been used with other types of paraffins (*13, 14*).

MATERIALS AND METHODS

The apparatus "Fractovap" Model B (Carlo Erba) was used with a thermal conductivity cell and the apparatus "Fractovap" Model C with a hydrogen flame ionization detector. The Model B apparatus is provided with an attachment for use at high temperatures; the attachment includes a device for the CO_2 oxidation of the substances previously chromatographed.

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[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

The columns used in the device for high temperatures are made of copper pipe with a 4 mm inside diameter, filled with 60- to 80-mesh Celite B.D.H. as the solid support and 1 meter of 10 or 15 percent Apiezon L as the stationary phase. The columns for the Model C apparatus are of stainless steel filled with 80- to 100-mesh Chromosorb W. as the solid support and 10 percent Apiezon L as the stationary phase.

The pure substances used as standards are specified below:

Tridecane	Light
Octadecane	Fluka
Docosane	Light
Octacosane	Eastman Kodak
Dotriacontane	Eastman Kodak

PROCESS

In preparation of the analysis materials we followed, with some modifications, methods of Cuzin *et al.* (*7*) and of Stedman and Rusaniwskyj (*10*).

Fermented Tobacco Wax

After 650 g of tobacco similar to a blend normally used in a standard brand of Italian cigarettes was extracted in a Soxhlet apparatus by benzene, we performed the following operation. The neutral phase was separated from the alkaline and acid phases. The residue was extracted by means of boiling ethyl alcohol, and the solution was cooled to −5° C. Two recrystallizations were done from ethyl alcohol, and the residue of the second was picked up in a cyclohexane and passed through an alumina column as described by Cuzin. The extracted paraffins were not passed through a charcoal column, but instead were recrystallized twice from ethyl alcohol. In this way a perfectly white product was obtained.

For 25 hours 370 g of the same tobacco blend was extracted in a Soxhlet apparatus with 750 ml of *n*-hexane. The process proposed by Stedman was then applied and was followed by two recrystallizations from ethyl alcohol to −14° C. Perfectly white paraffins were obtained by this process also.

Cigarette-Smoke Wax

One thousand five hundred cigarettes manufactured with a second lot of the same tobacco blend were smoked by an automatic machine under standard conditions: interval between puffs, 60 seconds; duration of a puff, 2 seconds; volume of a puff, 35 ml.

The condensate of the smoke was dissolved in 500 ml of methanol, the solvent was evaporated, and the residue was dissolved in benzene or *n*-hexene.

The subsequent processes are analogous to those used for the tobacco paraffins except that, in the second, further chromatography on an alumina column was performed.

The extraction was done with 250 ml of cyclohexane, the solvent was removed in a steam bath, and the residue was re-extracted with ethanol. In this case two crystallizations at −14° C were performed.

ANALYSIS

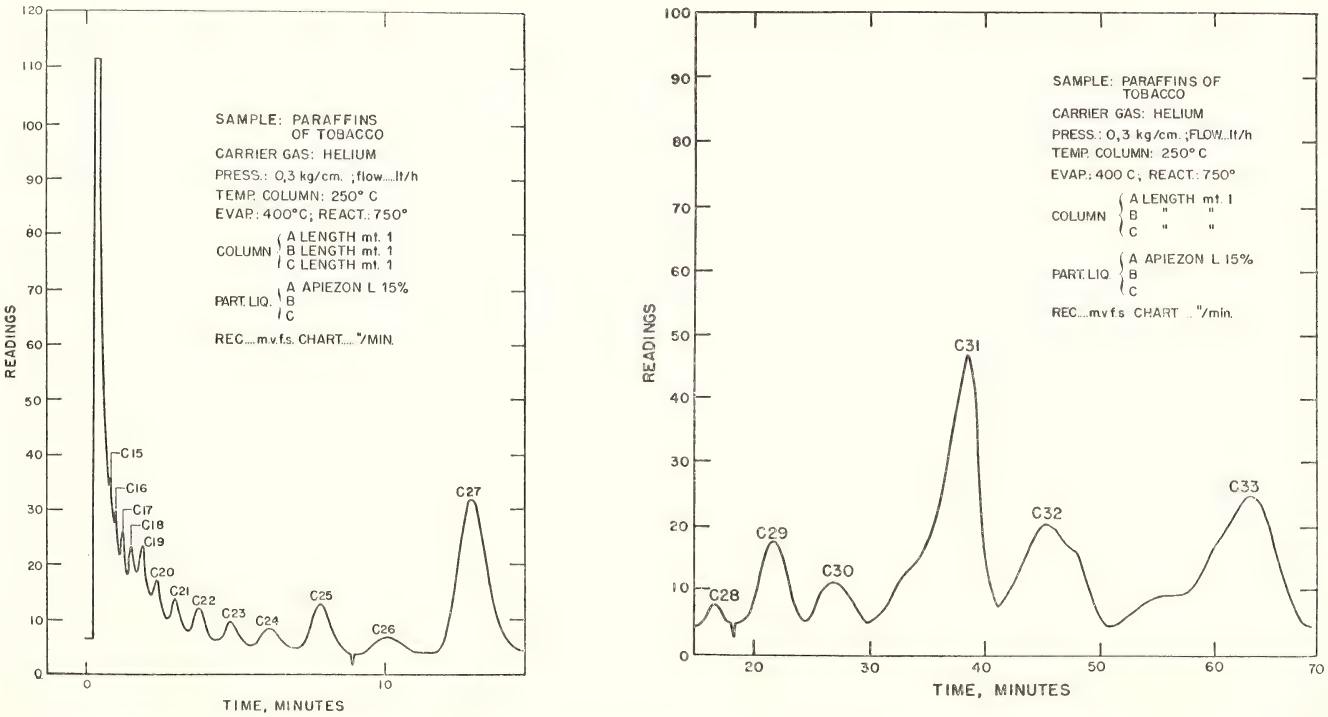
The paraffin samples were brought to melting temperature and introduced into the apparatus with a Hamilton syringe. For tests with the thermal conductivity cells, the cell temperature was 5° C, the combustion furnace temperature was 750° C, the carrier gas was helium, and the sample size was 0.02 to 0.05 ml. For tests with the flame ionization detector, the carrier gas was nitrogen and the sample size was 0.05 µl. For all tests the outlet pressure was atmospheric. Other test conditions were as follows:

	Thermal conductivity		Flame ionization		
	1	2	3	4	5
Injection temperature, °C	400	400	360	390	280
Column temperature, °C	230	280	230	255	253
Carrier gas flow, ml per min	24	31	18	25	25
Inlet pressure, mm Hg	456	532	684	852	852
Analysis time, min	60–70	30	53	173	15
Solid support	Celite (60–80 mesh)	Celite (60–80 mesh)	Chromosorb W. (80–10 mesh)		
Stationary phase	Apiezon L				
	(15%)	(10%)	(10%)	(10%)	(10%)

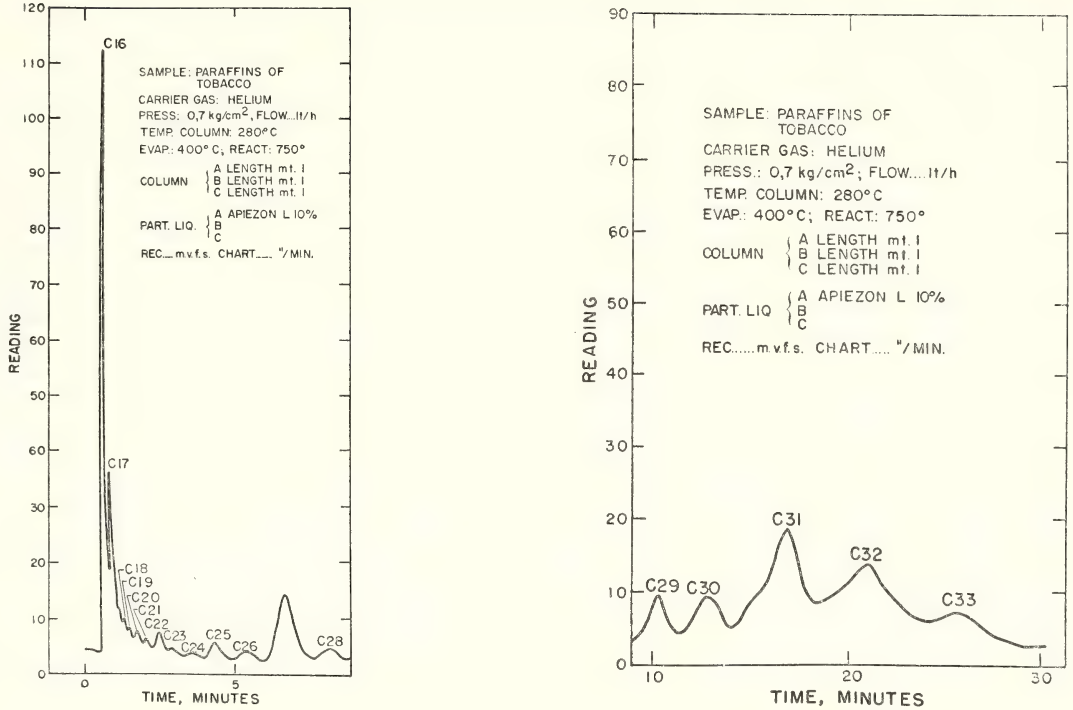
RESULTS AND DISCUSSION

Text-figures 1 and 2 show the chromatograms obtained by means of a high-temperature gas chromatograph with a thermal conductivity detector; text-figures 3, 4, and 5 show the chromatograms obtained with the flame ionization detector apparatus.

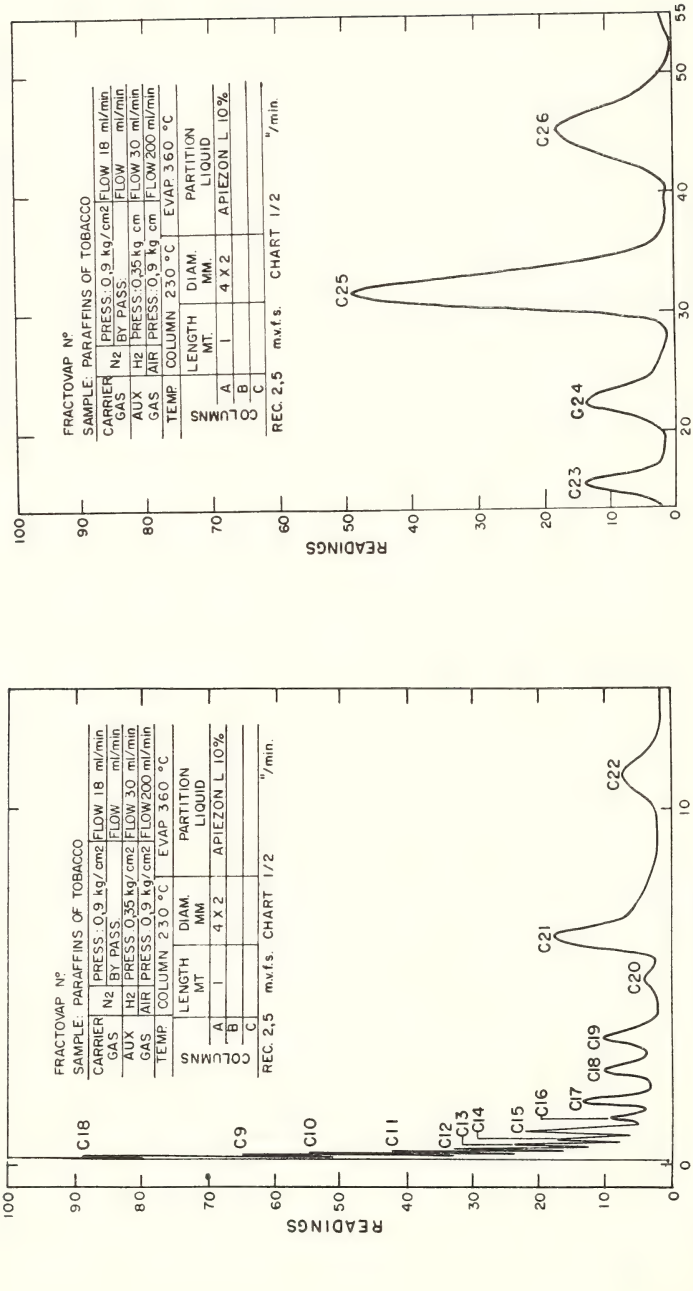
The complexity of the composition of paraffins extracted from tobacco and tobacco smoke was confirmed. The presence of paraffins of a very low melting point and of liquid paraffins at ordinary temperatures was ascertained by means of gas chromatography. These paraffins correspond to hydrocarbons having a low number of carbon atoms starting from C_8. We acheived the analysis of the tobacco paraffins by (text-fig. 3) working at a lower temperature (230° C) and by exploiting the high sensitivity obtained with the flame ionization detector. The presence of considerable quantities of isoparaffins was determined also. It should be noted that in all the analyses the peaks corresponding to C_{30} and to C_{32} do not consist



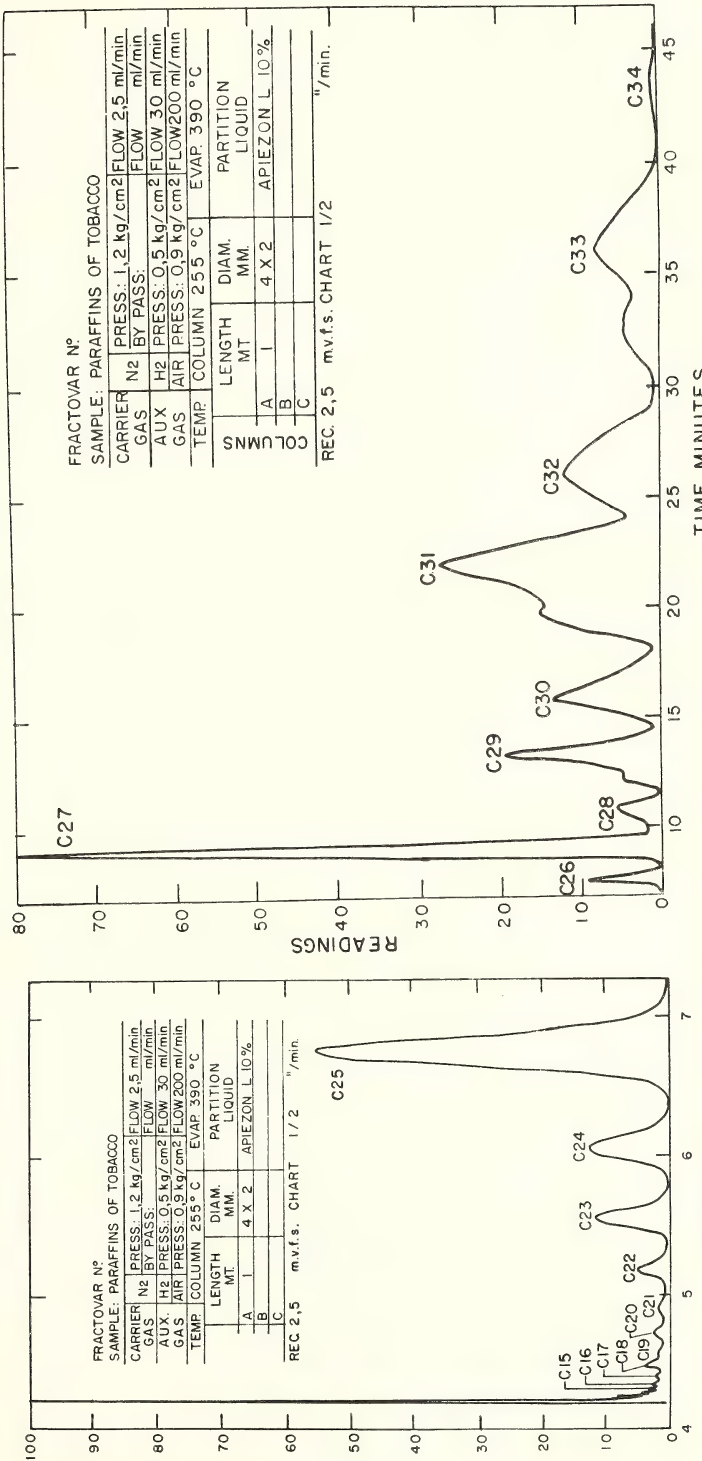
Text-figure 1.—Gas chromatography of paraffins of tobacco, *Apiezon L—15 percent*, thermal conductivity detector.



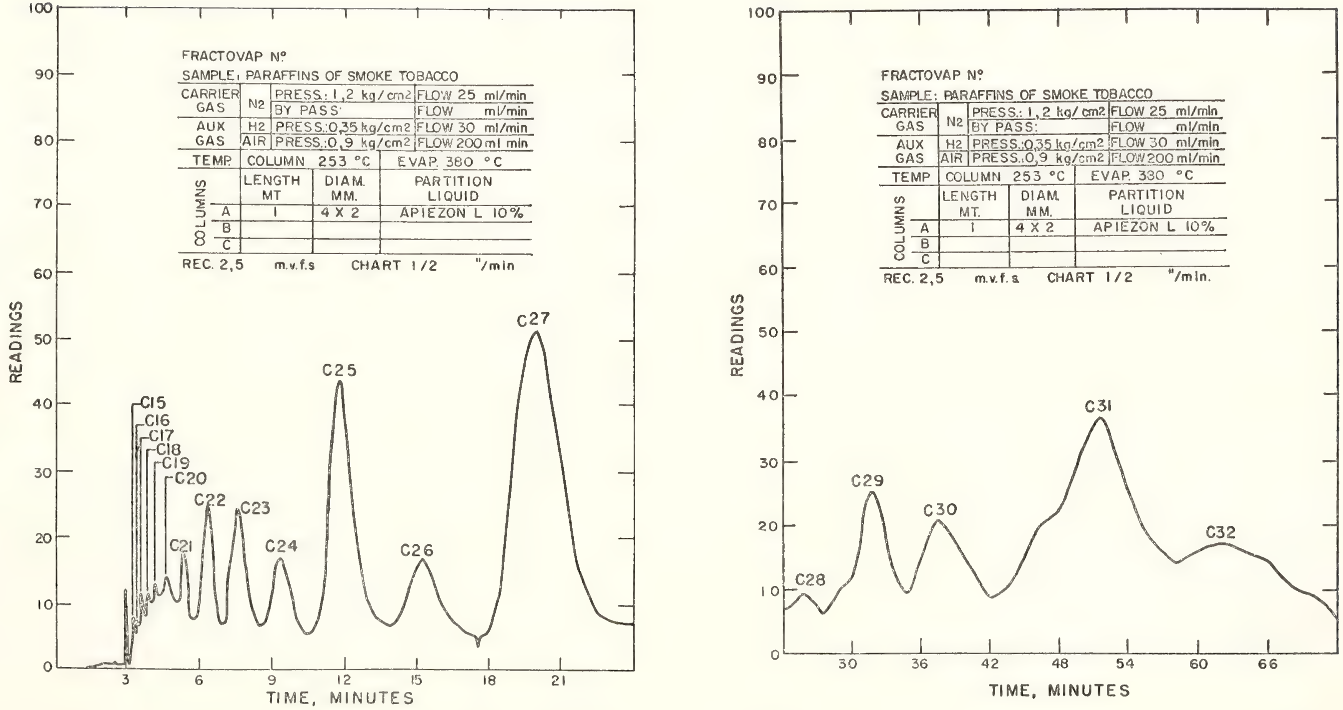
TEXT-FIGURE 2.—Gas chromatography of paraffins of tobacco, *Apiezon L—10 percent*, thermal conductivity detector.



Text-figure 3.—Gas chromatography of paraffins of tobacco, *column temperature, 230° C.* Flame ionization detector, *H_2 pressure 0.35 kg/cm^2.*



Text-figure 4.—Gas chromatography of paraffins of tobacco, *column temperature, 255° C.* Flame ionization detector, *H_2 pressure, 0.5 kg/cm²*.



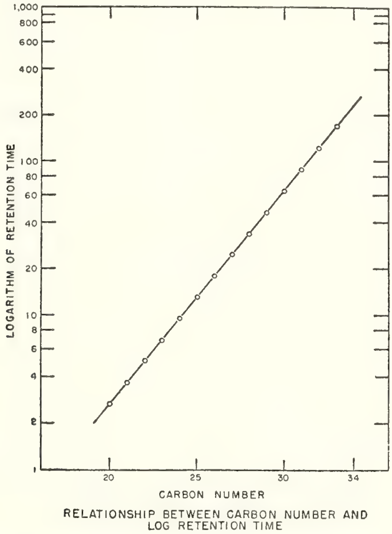
TEXT-FIGURE 5.—Gas chromatography of paraffins of tobacco, *column temperature 255° C.* Flame ionization detector, *H_2 pressure, 0.35 kg/cm²*.

mainly of *n*-paraffins. To confirm this, the identification of peaks has been based on the data of retention times of some pure compounds as docosane, octasane, and dotriacontane. The retention time (table 1) of all *n*-paraffins has been determined, and the relation between the logarithm of the retention volume and the number of carbon atoms has been verified. A straight line was obtained (text-fig. 6).

TABLE 1.—Relative retention volumes of *n*-paraffins*

Pentadecane	0. 026	Pentacosane	0. 522
Hexadecane	0. 038	Hexacosane	0. 723
Heptadecane	0. 048	Heptacosane	1. 00
Octadecane	0. 064	Octacosane	1. 36
Nonadecane	0. 085	Nonacosane	1. 67
Eicosane	0. 093	Triacontane	2. 57
Heneicosane	0. 133	Hentriacontane	3. 66
Docosane	0. 210	Dotriacontane	4. 81
Tricosane	0. 283	Tritriacontane	6. 63
Tetracosane	0. 388		

*Reference substance, heptacosane V_R rel=1; stationary phase, 10 percent Apiezon L; temperature, 255° C.



TEXT-FIGURE 6.—Relationship between carbon number and logarithm of retention time.

In the chromatogram of the paraffins of tobacco smoke a smeared background appears under the peaks; this may be due to the presence of foreign substances not fully separated in the crystallization phase. Paraffinic hydrocarbons from C_{14} to C_8 are present in fermented tobacco. A diminution of tritriacontane also was observed; further analyses are under way to perfect the methods of separating and of determining the paraffins.

Table 2 lists the composition of tobacco and tobacco-smoke paraffins as percentages. In determining the composition of cigarette-smoke wax, we deducted the area due to the smeared background. The percentage of the isoparaffins was also determined approximately in respect to *n*-paraffin for the peaks corresponding to C_{29}, C_{31}, and C_{33}; the values are 16, 22.2, and 24.5 percent, respectively.

TABLE 2.—Composition of tobacco and tobacco-smoke paraffins—percentage

Paraffins	Percent composition	
	Tobacco	Smoke tobacco
C_8	Traces	—
C_9	Traces	—
C_{10}	Traces	—
C_{11}	Traces	—
C_{12}	Traces	—
C_{13}	Traces	—
C_{14}	Traces	—
C_{15}	0. 029	Traces
C_{16}	0. 013	Traces
C_{17}	0. 042	0. 056
C_{18}	0. 057	0. 049
C_{19}	0. 057	0. 029
C_{20}	0. 058	0. 132
C_{21}	0. 018	0. 179
C_{22}	0. 086	0. 417
C_{23}	0. 186	0. 460
C_{24}	0. 333	0. 350
C_{25}	1. 463	1. 940
C_{26}	0. 654	0. 899
C_{27}+iso	7. 780	10. 350
C_{28}+iso	0. 992	1. 560
C_{29}+iso	9. 260	10. 280
C_{30}+iso	9. 004	11. 20
C_{31}+iso	36. 56	39. 95
C_{32}+iso	16. 84	17. 96
C_{33}+iso	16. 60	4. 567
C_{34}+iso	0. 347	—

CONCLUSIONS

This work has demonstrated: 1) that high-temperature gas-liquid chromatography can be used particularly in the search and determination of paraffins of tobacco and tobacco smoke; 2) that paraffinic hydrocarbons with a low number of carbon atoms are present as well as considerable quantities of isoparaffins; 3) that the complete analysis is rapid and eliminates any fractionation of paraffin waxes for determination of single paraffinic hydrocarbons.

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DISCUSSION

DR. HOFFMANN: In our experiments, long-chain paraffinic hydrocarbons show no promoting or carcinogenic effect, but do show a retardation effect. In other words, they limit the resorption through mouse skin of carcinogenic hydrocarbons so that a decrease in activity is obtained. A curve was obtained for benzo[*a*]pyrene in 0.005 percent concentration applied 3 times weekly. Waxes from tobacco-smoke condensate, with a melting point that varied a little—56 to 58 or 60, showed practically no tumor response. I think 1 mouse out of 30 got 1 tumor. We have not applied the tobacco-smoke condensate waxes at the same time; we did it on Tuesday and Thursday, 2 times weekly. Benzo[*a*]pyrene as the control was done on Monday, Wednesday, and Friday. We had the same idea, to have tobacco-smoke condensate waxes as the promoter, but in our work it turned out just the opposite. The tumor activity of benzo[*a*]pyrene, I think, has indicated a significant decrease. Thank you very much.

MR. FOX: I would like to ask the author if he used any other substrates than Apiezon L for the separation of the paraffins especially in the nonpolar synthesis such as SE-30 or other gum rubbers.

DR. CARUGNO: We used only Apiezon L.

DR. LYONS: I think preliminary purification of the wax mixture can be obtained by forming adducts with thiourea. It forms crystalline adducts. It could be used along with crystallization from methanol at −5° C.

DR. HOFFMAN: Dr. Lyons, the isoparaffins would be retained on the column while the paraffins would pass through. Is that not correct?

DR. LYONS: Yes.

Analysis of Coal Tar Products by High-Temperature Gas Chromatography [1]

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SUMMARY

Research at the Laboratories of the Société Carbochimique, Tertre, Belgium, since 1957 on the use of gas chromatography for the study of coal tars and derivatives is described. A very simple apparatus permits work at very high temperature (±400° C). This apparatus is constructed of pyrex glass, including the catharometers, which are equipped with tungsten wire as a sensing element. The stationary phases selected were silicon grease and polyester (Reoplex 400, Geigy). Examples are given of satisfactory results obtained in all tar products: raw tars, distilled oils, and road tars. Judicious choice of the stationary phase permitted analysis of the volatile components of the hard pitch, including unidentified products that boil at temperatures between 600 and 700° C. These techniques should aid in the analysis of air pollutants such as heavy aromatic hydrocarbons.—NCI Monogr. No. 9: 183–191, 1962.

THE ANALYSIS of complex mixtures of the aromatic hydrocarbons encountered in coal carbonization products such as tars, oils, and pitch is very difficult, if not impossible, by conventional means: fractional distillation, crystallization, liquid chromatography, chemical reactions, spectral analysis, etc. Furthermore, the samples used in these techniques must be of significant value, incompatible with the analysis of traces. High-temperature gas chromatography, for the first time, allows us to obtain very rapidly and with a small sample a fine picture of the volatile mixtures obtained by coal carbonization.

Since 1957, in the Laboratories of the Société Carbochimique, Tertre, Belgium, gas chromatography has been applied to the analysis of coal tar and its derivatives. As a result of these studies, gas chromatography is used in scientific and industrial applications in many European laboratories (Belgium, France, Holland, Great Britain, Germany, etc.).

APPARATUS

All work was performed with a chromatograph constructed in our laboratory (*1*, *2*). It is very simple, but remarkably useful for high-temperature work (up to ± 400° C). The column and the catharometers were made of pyrex glass; the whole apparatus was glass-blown after the U-shaped column was filled with the stationary phase. The entire assembly was situated in a horizontal insulated cylindrical furnace and was heated by a 1000-watt (maximum) electrical coiled resistor (text-fig. 1). The glass catharometers (text-fig. 2) were equipped with platinum filaments (20 μ by 10 cm; resistance at 20° C = 28 ohms) or with more sensitive tungsten filaments (10 μ by 10 cm; resistance at 20° C = 55 ohms). The catharometers were used in a conventional Wheatstone bridge (*3*) with current supplied by an 18-volt battery. The output current was directed to a multirange Philips recorder (1 mv at full scale). The sensitivity was very good, S = 200 ml × mv per mg, measured at the chrysene peak at a column temperature of 350° C (*3*), and no noise was observed even under the more severe conditions of use.

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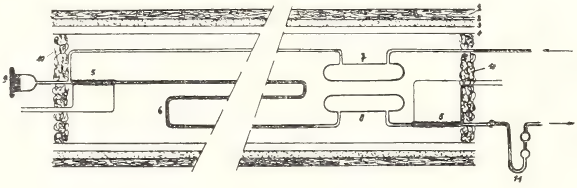
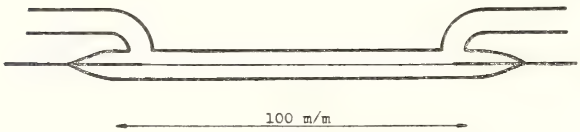
[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.



1. Asbestos.
2. Coiled heating resistor.
3. Pyrex tube.
4. Shielding foil.
5. 10-Watt auxiliary heater.
6. U-bended column.
7. Inlet catharometer.
8. Outlet catharometer.
9. Serum cap.
10. Glass wool.
11. Trap.

TEXT-FIGURE 1.—High-temperature gas chromatograph.



TEXT-FIGURE 2.—Glass catharometer.

Helium (99.8%) was supplied to the instrument through a precision two-stage pressure regulator. The supply was controlled by a mercury manometer at the head of the column, and its flow was measured by a soap-bubble meter; the flow rate was approximately 25 to 75 ml per minute, depending on the temperature of the column.

Samples of ± 10 mg in concentrated solution were introduced with a microsyringe through a serum cap; the vaporization zone, fitted with glass wool, was overheated (auxiliary resistor of 10 watts).

Eventually, the eluted products were trapped at the exit of the column in U-tube traps connected to the column by a spherical glass joint and filled with 0.5 ml of solvent. Straight tubes refrigerated with dry ice were occasionally used to trap solid products.

The apparatus was supplied with a heating current stabilized by a saturated transformer and regulated by a variable transformer. It was used up to 400° C with perfect stability of the baseline; no drift was observed, even at full sensitivity.

ANALYSIS OF TAR OILS AND TARS

Stationary Phase

The solid support chosen was Sil-O-Cell C22 insulating brick (Johns-Manville, U.S.A.) crushed and calibrated between 50 and 80 mesh for a column up to 3 meters long and between 30 and 50 mesh for a longer column up to 6 meters long.

A suitable nonpolar stationary phase was Dow-Corning High Vacuum Silicone Grease (30 wt % on the brick); it was generally used because of its stability at a very high temperature (a week at 350° C).

A 6-meter column heated at 360° C (flow rate of 40 ml per min) gives an efficiency of 3,500 theoretical plates, measured at the chrysene peak.

Reoplex 400, a polyester from Geigy, England, was eventually employed as polar liquid phase, but its use is limited to 275° C during a few days.

Trapping and Complementary Infrared Analysis

Liquid products eluted from the column were trapped in 0.5 ml of isooctane, a suitable solvent for infrared studies of aromatics between 10 and 15 μ (finger-prints region).

Solid products were generally trapped in a volatile solvent, such as benzene. The solution obtained was then poured, in an agate mortar, on 200 mg of finely crushed KBr; after the KBr and solvent were thoroughly mixed, the solvent was evaporated and the KBr was disc-pressed in the usual way. The obtained spectra were very pure and useful for qualitative or quantitative analysis. This technique was used intensively to resolve binary mixtures of products unseparated by chromatography, such as anthracene-phenanthrene mixtures (*2*).

RECORDING AND INTERPRETATION OF CHROMATOGRAMS

With the 6-meter silicone columns, 4 working temperatures were selected: 130, 185, 260, and 360° C. It was thus possible to analyze, step by step, a coal tar boiling up to 500° C. Fractionated oils, of course, were analyzed only on a column at suitable temperature.

Relative retention times of more than 80 tar constituents have been published elsewhere (*4*). The standards chosen were indene, naphthalene, and anthracene, which give well-resolved peaks, easily identifiable on the chromatograms of tar products.

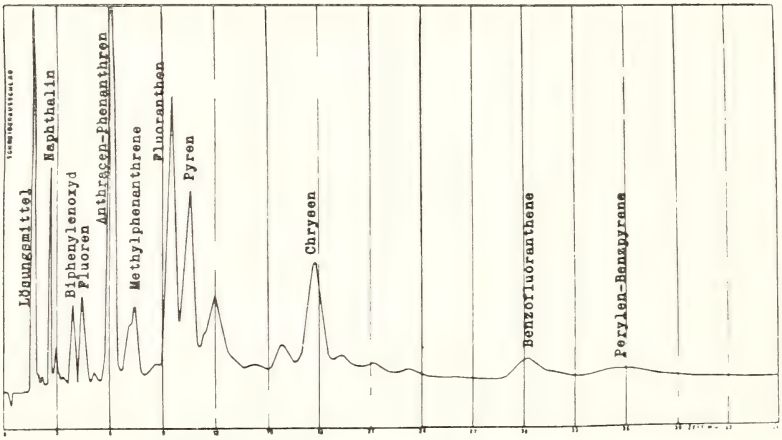
Since silicone grease is a nonpolar phase, unknown products were extrapolated from linear relationships between logarithms of relative retention times and boiling temperatures.

Infrared identification of trapped products was used, and confirmation finally was found on a second chromatogram of the sample after addition of suspected products.

Because of the complexity of the mixtures to be analyzed and because the exact nature of some minor peaks was unknown, the simplest well-known method of internal normalization was generally used.

Areas of peaks were determined by multiplication of the peak height by the retention time. This method was chosen to permit calculation of incompletely separated or overlapping peaks.

Very satisfactory results were obtained on synthetic mixtures (*1*, *2*). Numerous examples of analyses are given elsewhere: oils, tars, road tar, and pitch (*1*, *2*, *4*, *5*). Up to now, 50 peaks have been observed in the chromatogram of raw tar, giving all the components boiling up to 500° C (40 wt % of the tar). For example, text-figure 3 gives the chromatogram of an anthracenic oil.



TEXT-FIGURE 3.—Chromatogram of heavy anthracenic oil.

ANALYSIS OF HARD PITCH

A few pitch products boil at temperatures above 500° C but were undetectable with the described column because of broadening of peaks after 30 minutes of recording. It was also impossible to heat the column over 360 to 375° C because of the decomposition of the silicone grease.

Exploration of this region was attempted, however, by use of a more porous column. In fact, the stationary phase was only 10 percent of silicone grease on Sil-O-Cell C22 washed with aqua regia (*6*).

Apparatus and Procedure

Chromatograph: as described.

Column: 2.5 m by 6 mm.

Stationary phase: Dow-Corning High Vacuum Silicone Grease; 10 weight percent on Sil-O-Cell C22 (Johns-Manville, U.S.A.) washed with aqua regia and calibrated between 50 and 80 mesh.

Mobile phase: helium at 60 ml per minute at an inlet pressure of 600 mm Hg.

Temperature: between 300 and 360° C, depending on the sample.

Sample: ± 3 mg in concentrated solution.

Catharometers bridge: 18 volts, giving 0.2 amp at 320° C.

A freshly packed column must be stabilized overnight at 360° C and is usable for a week.

RESULTS

Table 1 shows the potentialities of the described column for the analysis of the heavy components of tar and pitch and retention times of heavy polynuclear hydrocarbons.

TABLE 1.—Retention times of heavy polynuclear hydrocarbons

	Absolute retention times (min and sec)			
	Column at 320° C		Column at 360° C	
Hydrocarbons	T_R absolute	T_R relative	T_R absolute	T_R relative
Anthracene	1:34	0.39	1:12	0.48
Fluoranthene	2:12	0.55	—	—
Pyrene	2:24	0.60	1:46	0.71
Chrysene	4:00	1.00	2:30	1.00
Benzo[*a*]pyrene	7:40	1.92	4:03	1.62
Picene	12:43	3.18	6:07	2.45
Pentacene*	13:50	3.46	5:45	2.30
	18:00	4.50	6:51	2.74
Coronene	—	—	10:48	4.32

*Two peaks.

Extrapolation of the relationship between logarithms of retention times and boiling temperatures indicates that a compound boiling at 750° C will be eluted in approximately 30 minutes (column at 360° C).

The technique described was used to study samples of our collection. Generally speaking, the samples were chromatographically pure except for a picene sample containing 10 weight percent of a compound that boiled ± 10° C lower than the picene and a pentacene sample that gave two peaks attributable to two products 40 and 60 weight percent, which boiled at 532 and 551° C, respectively.

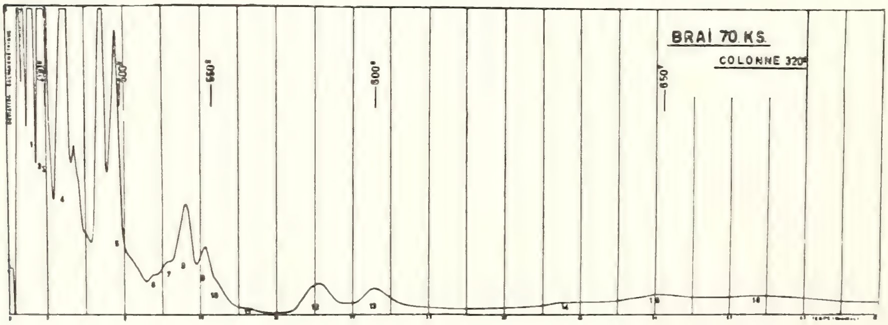
The boiling points and the retention times of well-described products can be used to estimate still undetermined boiling points or to clear discrepancies between values found in the literature. A few examples are given in table 2.

TABLE 2.—Estimated boiling points of some hydrocarbons at 760 mm Hg (° C)

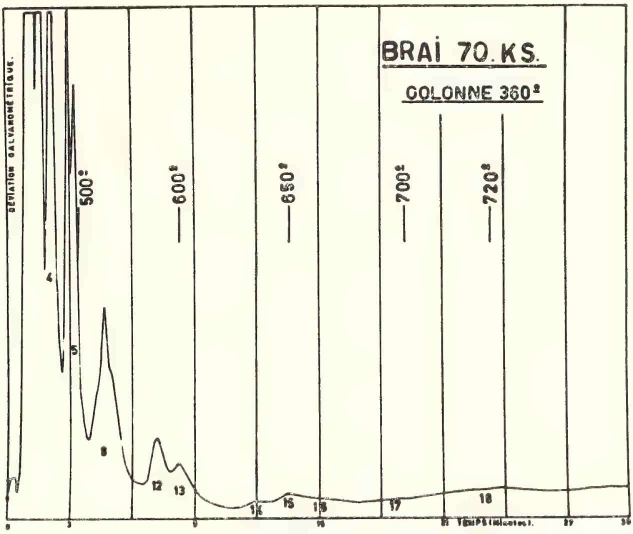
Hydrocarbon	Found in the literature	Found by chromatography
Perylene	503 (*7*) 500–510 (*8*) ± 460 (*9*) > 450 (*10*)	± 495
Picene	536 (*7*) ± 518 (*9*) 518–520 (*11, 12*) 519 (*8, 10*)	± 538
Coronene	525 (*12*)	± 602
Pentacene (2 peaks)	?	± 532 ± 551

Finally, it was possible to analyze the volatile components in hard-coal tar pitch. Two chromatograms were run at 320 and 360° C, respectively (text-figs. 4 and 5). The first one included components boiling up to 600° C, and the second one, heavier products, up to ±750° C. In these chromatograms, the products with boiling points below 400° C are unseparated and must be resolved in a third chromatogram run under the conditions chosen for oils (6-meter column, 30 wt% silicone, 300° C) (*5*).

In table 3, the complete gas chromatographic analysis of hard-coal tar pitch (70° K-S) and the probable boiling points of unknown products are given, as well as the estimated composition in weight percent. In the



TEXT-FIGURE 4.—Chromatogram of pitch (column at 320° C).



Text-figure 5.—Chromatogram of pitch (column at 360° C).

pitch, 41 components totaled 27.8 weight percent of the volatile compounds boiling up to 750° C.

Table 3.—Analysis of hard-coal tar-pitch components, boiling up to 750° C

Number	Component	Peak number in text-figure 4 or 5	Probable boiling point, °C	Weight percent
1	Indene			Trace
2	Naphthalene			0. 2
3	2-Methylnaphthalene			Trace
4	1-Methylnaphthalene			Trace
5	Acenaphthene			0. 4
6	Diphenylene oxide			0. 1
7	Fluorene			0. 2
8	?		300	0. 1
9	Methylfluorenes ?		305	Trace
10	Benzothionaphthenes		331	0. 1
11	Anthracene	1		0. 2
12	Phenanthrene			1. 5
13	Carbazole			0. 4
14	2-Methylphenanthrene ?		353	0. 1
15	Methylanthracenes ?		358	0. 3
16	Methylcarbazoles ?		368	0. 1
17	Fluoranthene	2		2. 2
18	Pyrene	3		1. 9
19	?		398	1. 2
20	Benzofluorenes ?		405	0. 1
21	?		430	0. 6
22	Chrysene	4		3. 3
23	Tetracene ?		452	0. 7
24	?		460	0. 6
25	?		469	0. 2
26	Benzofluoranthenes		484	2. 7
27	?		488	1. 1
28	Perylene-benzopyrenes	5	495	2. 2
29	?	6	520	0. 2
30	?	7	528	0. 5
31	Picene	8	536	1. 5

TABLE 3.—Analysis of hard-coal tar-pitch components, boiling up to 750° C—Con.

Number	Component	Peak number in text-figure 4 or 5	Probable boiling point, °C	Weight percent
32	?	9	546	1. 1
33	?	10	553	0. 5
34	?	11	564	0. 1
35	?	12	586	0. 8
36	Coronene ?	13	600	0. 7
37	?	14	636	0. 2
38	?	15	650	0. 7
39	?	16	660	0. 6
40	?	17	694	0. 1
41	?	18	720	0. 3
Total				27. 8

CONCLUSIONS

High-temperature gas chromatography can be used with success to analyze coal tar and its derivatives, including heavier products such as polynuclear compounds up to 7 rings. The exploratory work described gives a good idea of the potentialities of the method, which will undoubtedly be improved by innovations such as capillary columns and temperature-programmed chromatography. Such methods are now used on tar oils at the Coal Tar Research Association (England) (*13*). The application of high-temperature chromatography with ionization detectors to polynuclear hydrocarbons was recently described by Gudzinowicz and Smith (*14*).

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Comparison of Aromatic Polycyclic Hydrocarbons From Gasoline Engine and Diesel Engine Exhausts, General Atmospheric Dust, and Cigarette-Smoke Condensate [1]

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SUMMARY

Exhaust particulates from gasoline and diesel-powered vehicles and a sample of general atmospheric soot collected over a 3-month winter period in industrialized central Glasgow, Scotland, were analyzed for aromatic polycyclic hydrocarbons. The results were compared with a similar analysis of cigarette-smoke condensate. Whereas the different source materials yielded essentially similar qualitative patterns, wide quantitative differences were found.—NCI Monogr. No. 9: 193-199, 1962.

THE HYPOTHESIS that implied an exclusively causal relationship in the *pari passu* urban industrialization and lung cancer increase is no longer consistent with the majority of epidemiological findings, which implicate the habit of cigarette smoking as the most significant causative factor. Demographically, however, wide variations in incidence patterns exist and among the more reproducible findings is the greater incidence in regions of higher urban air pollution. An approach to the breakdown of this so-called "urban factor" is the subject of the present communication. The proposition that agents etiologically relevant to lung cancer have been introduced and/or have increased numerically in urban atmospheres over the recent past is examined. The investigations relate more to the British (and generally European) experience than to the American.

The possibility that the change from solid to liquid fuels in industry and in the home and the increased use of motor fuel were attended by increased lung cancer hazards has been indicated by a number of workers, notably Kotin's group. More specifically, the work of Mills and Porter (*1*) and Mills (*2*) affords evidence linking lung cancer and the inhalation of motor exhaust products. In Great Britain, Kennaway and Kennaway (*3*) pointed out that air pollution by coal smoke from domestic chimneys and boiler furnaces had been diminishing over a number of years, contraindicating any importance that this urban factor component might have in the etiology of the increasing lung cancer incidence. The question was then posed whether air pollution incidental to the use of liquid fuels could compensate, and even progressively enlarge, the atmospheric pool of carcinogenic agents. It followed that attention was directed initially toward the analysis of aromatic polycyclic hydrocarbons in two important sources of pollution, namely, in the incomplete combustion products of gasoline and diesel engines. The results were compared with similar analyses of samples of particulates from the general atmosphere and cigarette-smoke condensate. Some of the work has previously been published: Lyons and Johnston (*4*) and Lyons (*5*, *6*).

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[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29-31 1961.

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MATERIALS AND METHODS

A sample of gasoline exhaust particulates was obtained from a 3-6L Ford V-8 pilot car during a 2-day period of normal city motoring by fixing hemp sacks to the twin exhaust pipes. The diesel exhaust sample was similarly collected from a stationary public omnibus operating without load. Although the exhaust pipes were not occluded and holes were punched in the necks of the sacks below their point of attachment to the pipe, it is nevertheless believed that the obstruction caused back pressure and engine inefficiency. Particulates were dried over phosphorus pentoxide. The general atmospheric soot sample was collected over a 3-month winter period on fiber glass filters in a ventilator shaft at a site in central Glasgow, an area of high atmospheric pollution.

Aliquots of 20 to 30 g of the different dried particulates were Sohxlet-extracted with fluorescence-free benzene. Following removal of the benzene, the extracts were taken up in petroleum-ether (60–80° C, bp) and layered on prepared columns of alumina (Spence, Grade H, 100–200 mesh) for adsorption chromatography performed according to conventional methodology. Repeated fractionation of subfractions on alternate alumina and silica gel columns was of value. A convenient way of chromatographically separating closely related, strongly adsorbed compounds —to which particular attention was paid in view of the hitherto paucity of information—involved the incorporation of acetone (from 2 to 10%) in the petroleum-ether eluent.

Individual fractions were screened by fluorescence and ultraviolet and visible absorption analysis. A Hilger Medium Quartz spectrograph was used for the fluorescence spectrography. Excitation radiation of 365 mμ was routinely employed. A slit-width of 0.1 mm and an aperture length of 4 mm were maintained throughout the screening procedure. The solvent was usually spectroscopic cyclohexane.

A Uvispek spectrophotometer was used for absorption analysis. Cyclohexane or benzene was the usual solvent. For the quantitation of individual compounds, conventional baseline technique was used, with definitive peaks at the longer wavelengths at optical densities within the range of 0.1 to 0.7. The compounds given in table 1 were detected. No doubt there are many other compounds formed which are not indicated in the tables. As a result of overlap in the fractionation procedure, the order of elution is only approximate at a number of points, and many spectra are incomplete because the compounds giving rise to them were not sufficiently purified.

TABLE 1.—Compounds detected in various atmospheric pollutants—(G), (D), (A), and (C)* samples

Spectral maxima (mμ)†	G	D	A	C	Compound
1. A: 320, 287, 276, 268 F: —	+	+	+	+	Naphthalene
2. A: 340, 324 F: —	+	+	+	+	Acenaphthylene
3. A: 376, 355, 338, 252 F: 378, 395, 420	+	+	+	+	Anthracene
4. A: 293, 274 F: —	−	+	−	+	Phenanthrene
5. A: 386, 365, 347, 260 F: 387, 407	+	−	−	−	Anthracene derivative I
6. A: 380, 359, 342, 326, 257 F: 382, 403, 428	+	+	+	−	Anthracene derivative II
7. A: 389, 368, 349, 258 F: 390, 412, 440	+	−	−	−	Anthracene derivative III
8. A: 372, 262, 256, 335, 272 F: —	+	+	+	+	Pyrene
9. A: 359, 342, 288, 277 F: —	+	+	+	+	Fluoranthene
10. A: 377, 342, 328, 276, 264 F: —	+	−	−	+	Alkylpyrene
11. A: 376, (368), 355, 338, 324, 310, 291, 278 F: —	+	+	+	−	Orange compound (pyrene derivative?)
12. A: 388, 362, 346, 264 F: 388, 410, 435	+	−	−	−	Anthracene derivative IV
13. A: 386, 360, 348, 336, 292, 274 F: 386, 403, 428	+	−	−	+	Anthracene derivative V
14. A: 385, 359, 344, 290, 280 F: 385, 404, 428	+	+	+	+	Benz[*a*]anthracene
15. A: 362, 344, 266, 258 F: —	+	−	+	+	Chrysene
16. A: 382, 361, 330, 288 F: 382, 407, 431	−	−	+	−	Unidentified
17. A: 366, 332, 317, 290 F: —	+	+	+	+	Benzo[*e*]pyrene
18. A: 436, 411, 386 F: 437	+	+	+	+	Perylene
19. A: 403, 386, 365, 347, 298 F: 403, 426, 454	+	+	+	+	Benzo[*a*]pyrene
20. A: 408, 387, 368, 303, 291 F: 408, 430	+	+	+	+	Benzo[*ghi*]perylene
21. A: 368, 350, 302, 275, 255 F: 428, 450	+	+	+	+	Benzo[*b*]fluoranthene
22. A: 432, 408, 310 F: —	+	+	+	+	Anthanthrene
23. A: 471, 441, 415, 393 F: 471	+	−	−	−	Tetracene
24. A: 428, 410, 388, 342, 305, 293 F: —	+	+	+	+	Coronene
25. A: 396, 385, 373, 322, 300 F: 396, 406, 417, 443	+	−	−	−	Dibenz[*a,h*]anthracene
26. A: 458, 424, 414, 385, 366, 341, 327, 303, 275 F: 458, 466, 492, 526	+	+	−	−	Unidentified
27. A: 454, 433, 403, 381, 332, 317, 303 F: 462	+	+	−	+	4-Dibenzo[*a,l*]pyrene

See footnotes at end of table.

TABLE 1.—Compounds detected in various atmospheric pollutants—(G), (D), (A), and (C)* samples—Continued

Spectral maxima (mμ)†	G	D	A	C	Compound
28. A: 401, 380, 360, 309, 296 F: 401, 410, 428, 456	+	+	+	+	Benzo[*k*]fluoranthene
29. A: 388 F: 388, 410, 435	−	−	+	+	Unidentified
30. A: 413, 405, 381, 362, 345 F: 414, 437	+	+	+	+	Unidentified
31. A: 424, 411, 399, 377, 360, 349 F: 423	+	+	−	−	Pentaphene
32. A: 405, 391, 380, 340, 319, 309 F: 405, 430	+	−	−	−	Unidentified naphtho(2,3,1,2)pyrene?
33. A: — F: 435, 464	−	−	+	−	Unidentified
34. A: 433, 407, 326, 313, 300, 289 F: 434	+	−	−	−	Dibenzo[*a,l*]naphthacene
35. A: — F: 410, 435	+	−	−	−	Unidentified
36. A: 452, 424, 402, 314, 300 F: 451	+	−	−	−	Dibenzo[*a,h*]pyrene
37. A: 428, 416, 395, 378, 327, 306, 300 F: 416, 426, 430, 440	+	−	−	−	Dibenzo[*a,e*]pyrene
38. A: 405, 391, 377, 357, 344, 309 F: 405, 418, 428, 443, 453	+	−	−	−	Dibenzo[*b,pqr*]perylene
39. A: 396, 369, 347, 328, 304 F: 450, 467, 478	+	−	−	−	Unidentified
40. A: 422, 412 F: 422, 445	+	−	−	−	Unidentified
41. A: — F: 407, 432	−	+	−	−	Unidentified
42. A: 393, 382, 365, 335, 328, 313, 303, 287 F: 393, 415, 442	−	−	+	−	Unidentified
43. A: — F: 397, 418	−	−	+	−	Unidentified
44. A: 402, 369, 300, 282 F: 402, 417, 430	−	−	+	−	Unidentified
45. A: 352 F: 352	−	−	+	−	Unidentified
46. A: 380, 360, 352, 337, 320, 297, 272, 264 F: 380, 387, 399, 421	−	+	+	−	(Dibenzofluorene?)
47. A: 378, 352, 265 F: 378, 398	−	−	+	−	(Dibenzofluorene?)
48. A: 391, 383, 366, 348, 306, 290 F: 391, 414, 440	+	−	−	−	Unidentified
49. A: 385, 363, 347, 336, 312 F: 385, 407, 334	+	−	−	−	Tribenzo[*h,rst*]pentaphene
50. A: 397 F: 397, 422, 445	−	−	+	−	Unidentified
51. A: 412, 388, 365, 318, 296 F: 412, 435, 465	−	+	−	−	Indeno-1,2,3-fluoranthene?
52. A: 438, 414, 300, 290 F: 441, 470	+	−	+	−	Unidentified
53. A: 400, 390, 378, 356, 327, 312, 298 F: 400, 425, 445	+	−	−	−	Unidentified

*G: gasoline-soot sample; D: diesel-soot sample; A: general atmospheric-soot sample; C: cigarette-smoke condensate.

†A: absorption; F: fluorescence.

RESULTS AND DISCUSSION

Qualitatively, an expected but nonetheless interesting fact emerged: Many compounds were common to the vehicular exhausts and cigarette smoke. In the exhausts a variety of compounds possessing the anthracene stem was noted. The polycyclic hydrocarbons occurring in highest concentration in the 3 soot extracts appeared to lie in the 2 to 5 condensed-ring range.

Of the compounds detected, the following were carcinogenic to experimental animals: benzo[*a*]pyrene, benzo[*b*]fluoranthene, dibenzo[*a,h*]pyrene, dibenzo[*a,l*]pyrene, dibenzo[*a,e*]pyrene, dibenz[*a,h*]anthracene; and weak activity is attributed to benz[*a*]anthracene and benzo[*e*]pyrene. Further carcinogenic activity may well reside in the gasoline soot fractions, #30, 48, and 49, since carcinogenic fractions isolated from horizontal retort tar by Berenblum and Schoental (*7*) showed similar fluorescence peaks and chromatographic sequence.

Some hydrocarbon levels of the soots in parts per million are given in table 2.

TABLE 2.—Some hydrocarbon levels in soot samples (ppm)

Compound	Atmospheric soot	Diesel soot	Gasoline soot
Anthracene	73	36	96
Pyrene	221	292	110
Benz[*a*]anthracene	23	34	45
Benzo[*a*]pyrene	139	12	390
Dibenzo[*a,l*]pyrene	—	10	6
Benzo[*k*]fluoranthene	10	59	12

Noteworthy features are the disproportionately high pyrene level in the diesel exhaust sample, the contrasting levels of benzo[*a*]pyrene in the gasoline and diesel exhaust samples, and the absence from the general atmospheric soot sample of the two dibenzopyrenes.

Similarly high levels of benzo[*a*]pyrene have been found more recently in the exhaust of a gasoline-driven vehicle on a city driving schedule (229 and 295 ppm benzene solubles) and Kotin (*8*) found 310 μg per g of soot from an idling gasoline engine exhaust. Except for periods of high air pollution in some cities, the benzo[*a*]pyrene levels of general atmospheric particulates [*see* data by Kotin (*8*) and Commins, Waller, and Lawther (*9*)] rarely reach such levels. Gurinov, Zore, Ilina, and Shabad (*10*) demonstrated that for a selected number of towns in the USSR those in which liquid fuel was principally used—as against coal, peat, and wood in other towns—showed the highest concentration of airborne benzo[*a*]pyrene. The appreciable level of this compound in the air (152–255 ppm) of urban Reykjavik, Iceland, is thought to arise largely from liquid fuel effluents.

Both the gasoline and diesel exhaust particulates contain considerable quantities of adsorbed or encapsulated oil. In the gasoline exhaust sample, the ratio of oil (unburnt fuel and lubricant) to benzo[*a*]pyrene was found to be 100 to 0.16; the benzopyrene existed on carbon particulates in an oily solution at a concentration of 0.16 percent. This fact is interesting in the light of Horton, Denman, and Trosset's work (*11*), which showed that relative accelerating carcinogenic activity up to 2.5-

fold was obtained when benzopyrene and methylcholanthrene in paraffin solutions containing the agents at 0.04 to 0.20 percent were administered to mice.

Some points made by Tebbens *et al.* (*12*) are relevant to the present discussion. These workers found that the products obtained by the combustion of gaseous and simple aliphatic liquid fuels in the laboratory were similar to those obtained from a sample of gasoline exhaust; the same compounds were produced regardless of the fuel, but relative concentrations varied. The variables relating total soot to benzene extractible material were stated to be unknown, but little relationship was indicated. In the present work it would also appear that individual polycyclics, whole organic fractions, and graphitic carbon fractions of exhaust soots are related to independent variables. This conclusion is supported by the work of Schalla and MacDonald (*13*) and Gaydon and Fairbairn (*14*), who argue that graphitic carbon must be formed by a mechanism (stripping and subsequent hydrocracking followed by simultaneous polymerization and dehydrogenation on C or C_2 nuclei) different from the polymerization mechanism involved in the pyrosynthesis of aromatics.

Since the cigarette-smoke condensate data are not altogether germane to the subject matter of this symposium, it is sufficient to remark on the number of polycyclics common to the condensate and the exhausts and atmospheric dust. The concentration levels in the former were, relatively, of a much lower order.

CONCLUSIONS

The constellations of aromatic polycyclic hydrocarbons in samples of gasoline exhaust and diesel exhaust were compared to a sample of general atmospheric dust. The importance of the gasoline over the diesel exhaust as a possible source for the enrichment and enlargement of the atmospheric pool of the traditional carcinogens was shown.

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DISCUSSION

MR. HOWERTON: Did you observe any fluorescence for coronene?

DR. LYONS: No, I do not think I did. I know it is fluorescent although I have not obtained fluorescence spectra of coronene.

MR. HOWERTON: Did you attempt a vibrational analysis of the data?

DR. LYONS: No.

MR. HOWERTON: You had one compound which showed absorption at 424 mμ with fluorescence at 400?

DR. LYONS: Yes, such things can happen, can they not?

MR. HOWERTON: Only if it is anti-Stokes fluorescence. Is this just an anomaly or perhaps a misprint?

DR. LYONS: If the long wavelength absorption peak is very weak, I have found that the comparable mirror-image fluorescence band cannot be found in the spectrum.

MR. HOWERTON: You would expect if there is absorption at 424 mμ, there would be emission also at 424 mμ or very close to that. You did not observe it because it was so weak. Is that the explanation?

DR. LYONS: Yes.

DR. FALK: The question came up again in your very nice presentation whether there was a discrepancy between soot production and hydrocarbon production, whether they run parallel or not. We actually studied the elution of polycyclics from soot of different particle sizes, and got a completely different pattern, depending on the average particle size of the soot that we were working with. If the particle size is between 10 and 30 mμ and the usual solvent is used, you do not obtain any polycyclic hydrocarbons whatsoever, even though they are there. We know that they are there because we put them there. If you take a particle size of an average range between 30 and 50 mμ and extract these particles, you will obtain the three- and four-ring hydrocarbons but not the larger ones. With particle-size ranges between 60 and 80 mμ, you can obtain everything up to coronene. We also investigated particle sizes up to 450 mμ.

DR. KATZ: About this question of the relation between soot or carbon and polycyclics, we have investigated samples of carbon black prepared for the rubber industry. This carbon black was a prolific source of polycyclic hydrocarbons, even up to the size of coronene. We also obtained indications that some of the polycyclics were very strongly absorbed on the soot, carbon black.

Analysis for Airborne Particulate Hydrocarbons: Their Relative Proportions as Affected by Different Types of Pollution [1]

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SUMMARY

This paper is divided into two main sections, Methodology and Results of Analysis. In the Methodology section, general analytical methods and the more highly selective methods that have been found to be of some use in air pollution research are discussed. In the section on Results of Analysis of various airborne particulate samples, the concentrations of polycyclic aromatic hydrocarbons in urban and non-urban atmospheres are considered. The influence of various types of pollution, such as fumes from coal combustion, auto exhaust, and hot-coal tar pitch, on the relative proportions of various polycyclic hydrocarbons in the air was investigated. The hydrocarbon composition of some types of source samples also was investigated.—NCI Monogr. No. 9: 201–220, 1962.

METHODOLOGY

MUCH WORK is needed to develop new methods for air pollution analyses and to improve the older ones. Two main types of analytical techniques are required: methods for research investigations and for routine determinations that could be useful in understanding air pollution and its problems. A more thorough investigation of the shortcomings of collection, separation, and analytical methods is needed before routine analysis for atmospheric pollutants can be considered completely satisfactory. Shortcomings can be reduced significantly by use of reproducible procedures that make comparisons more valid.

In this section some of the newer, useful techniques employed in our investigation are discussed:

1. Piperonal chloride, 3-nitro-4-dimethylaminobenzaldehyde (NDB), and isatin procedures

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[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

[2] This study was accomplished with the able assistance of the author's colleagues, Walter Elbert, Francis T. Fox, Thomas R. Hauser, James Meeker, James L. Noe, Thomas W. Stanley, and Ronald Wilson. The author is indebted to Elbert C. Tabor and the National Air Sampling Network Staff for the benzene extractions of particulate samples; to Robert L. Stenburg, Darryl J. von Lehmden, and Robert P. Hangebrauck for collection of the samples from pollution sources; and to Ralph Larsen and his staff for the collection of samples from auto tunnels.

2. 3-Methyl-2-benzothiazolone hydrazone (MBTH), 5-nitroisatin, and 4-azobenzenediazonium fluoborate (ADF) procedures
3. Procedures for compounds containing the cyclopentadiene CH_2 group
4. Procedures for compounds containing the pyrrol group
5. Selective procedures for pyrene and anthracene compounds
6. Nondestructive spectrophotometric procedures
7. Nondestructive spectrophotofluorometric procedures

1. *Piperonal chloride, NDB, and isatin procedures.*—The reagent, piperonal chloride, reacts readily with all aromatic compounds that have their highest electron density on an unsaturated carbon atom and which are more basic than benzene (*1–3*). Consequently the reagent will react with aromatic or aralkyl hydrocarbons and their halide derivatives, phenols, diaryl and aralkyl sulfides, diaryl and aralkyl ethers, aromatic acylamines, and heterocyclic compounds, where the ring hetero atom is oxygen, sulfur, selenium, or the imino group. Aromatic compounds containing electronegative groups, such as a quinone, nitro, or ammonium substituent, give negative results in the test, which has been used in estimation of the aromatic content of the benzene-soluble fraction of airborne particulates (*3*). With this test the aromatic hydrocarbon content of various atmospheric particulates can be quickly fingerprinted. The complexity of the major aromatic compounds as well as their quantity can be estimated. A mixture of the simpler aromatic compounds that reacted in the procedure would absorb at much shorter wavelength than a mixture of the larger and more basic aromatic compounds; in addition the amount of benzo[*a*]pyrene in the air can be estimated.

Several additional procedures for the spectrophotometric characterization and determination of polycyclic hydrocarbons have been described (*4, 5*). The new reagents, NDB and isatin, react only with the more basic polycyclic hydrocarbons, especially the pericondensed aromatic hydrocarbons. With these reagents, wavelength maxima for the various reactive compounds range from 480 to 965 mμ (*1, 4, 5*).

2. *MBTH, 5-nitroisatin, and ADF procedures.*—Whereas the techniques just described can be used for the analysis of compounds ranging in basicity from the alkylbenzenes to the diarylamines, those with MBTH (*6*), 5-nitroisatin (*7*), and ADF (*8*) can be used for the analysis of compounds ranging in basicity from the diarylamines to the aromatic amines. Thus, these two methods overlap somewhat. Of the polynuclear aromatic hydrocarbons only azulene reacted with these reagents (*9, 10*). Other polynuclear azulenes containing the unsubstituted 1-position of azulene could also be expected to react, as could large polynuclear aromatic hydrocarbons with the same order of basicity as azulene.

Carbazole can be characterized or determined in the benzene-soluble fraction or airborne particulates with MBTH or *p*-nitrosophenol without prior separation (*11*). Other electrophilic reagents that can be used for the analysis of nucleophilic compounds of the same order of basicity as carbazole and azulene have been compared (*10*). Many of these methods could be applied for characterization and determination of the polynuclear carbazoles, some of which are known to be carcinogenic and conceivably could be present in the neutral oxygenated fraction of some airborne particulate samples.

3. *Procedures for compounds containing the cyclopentadiene CH_2 group.*—A thermochromic color test has been devised for fluorenic and polynuclear fluorenic compounds (*12*) and has been used for the characterization of fluorene and the benzofluorenes in column chromatographic fractions of airborne particulates (*13*).

Spot test and spectrophotometric procedures in which 1,2-dinitrobenzene is used for the detection and determination of a large number of compounds containing the cyclopentadiene CH_2 group have been applied to the analysis of airborne particulates (*14*). Compounds such as cyclopentadiene, indene, fluorene, 4*H*-cyclopenta[*def*]phenanthrene, and the benzofluorenes give positive results.

A third method that has been used only for the characterization of fluorenic and polynuclear fluorenic compounds depends on the ultraviolet-visible absorption spectra and the excitation and emission fluorescence spectra of these compounds in a strongly alkaline solution. The spectra obtained in this manner are unstable but very characteristic. With the help of these methods, as well as with chromatographic and nondestructive spectrophotometric and spectrophotofluorometric procedures, fluorene and the 3 benzofluorenes have been identified in air samples.

4. *Procedures for compounds containing the pyrrol group.*—In section 2, methods that could be used for analysis of compounds such as pyrrol, indole, carbazole, and the polynuclear carbazoles were mentioned. In addition to analysis by these methods, carbazole and polynuclear carbazoles containing an unsubstituted NH group can be readily characterized by means of their ultraviolet-visible absorption spectra and their excitation and emission fluorescence spectra in strongly alkaline solution. The spectra of these compounds in the neutral and alkaline solvents are very characteristic, although the spectra in alkaline solution are somewhat unstable.

5. *Selective procedures for pyrene and anthracene compounds.*—Highly selective detection procedures for pyrene (*15*) and anthracene (*16*) compounds are also available.

6. *Nondestructive spectrophotometric procedures.*—The main advantage of a destructive method of analysis, in which the compound analyzed is essentially destroyed, is that a quick, direct analysis of a mixture for one compound or a family of compounds can be made without extensive preliminary separation. In addition, such a procedure can be used terminally in characterization of an unknown. Where a fairly large family of compounds must be individually determined, however, a separation procedure of some type is necessary. Identification of individual compounds on the basis of their separation alone is usually questionable, and therefore supplementary techniques are necessary. Determination of the absorption spectra of the separated components of a mixture is one of the standard techniques, especially if the particular compounds absorb in the ultraviolet. This technique (*17*) has been applied to the separation,

characterization (*13*, *18*), and determination (*19*, *20*) of polynuclear aromatic hydrocarbons in airborne particulates. In addition aliphatic hydrocarbons can also be determined. In this procedure the benzene-soluble fraction is passed once through a chromatographic column, the aliphatic hydrocarbons in the first tube are determined by high-temperature gas chromatography, and the absorption spectrum is determined in each of the remaining tubes. Appropriate fluorometric studies, paper chromatography, and destructive color tests are used, if necessary, for characterization.

One curious result in column chromatography may be worth more thorough investigation. In several experiments the effect of 2,4,5,7-tetranitrofluorenone (15 mg/g alumina) dispersed in the alumina of the column has been studied in the chromatographic separation of the benzene-soluble fraction of airborne particulates. Such a column gave a benzo[*a*]-pyrene fraction minus benzo[*k*]fluoranthene. This benzo[*a*]pyrene fraction dissolved in pentane was identical spectrally from 374 to 450 mμ to a mixture of benzo[*a*]pyrene and perylene in the same concentrations.

For the identification of unknowns, paper chromatography of a fraction, followed by the determination of absorption and fluorescence spectra of the different spots, is frequently indispensable. However, the prime necessity is to develop the simplest possible procedure for the determination of the largest possible number of organic compounds in airborne particulates.

Our present procedure for the determination of hydrocarbons consists of the following steps: The benzene-soluble fraction of a particulate sample is passed through an alumina column. The aliphatic fractions contained in the first few tubes are combined, and then an aliquot of this material is passed through a gas chromatographic column for the analysis of the aliphatic hydrocarbons.

The contents of the remainder of the tubes are then examined spectrophotometrically and thus analyzed for about 8 polycyclic aromatic hydrocarbons by a baseline technique (*21*, *22*). The ultraviolet-visible absorption spectra of many polycyclic aromatic hydrocarbons have been reported (*23–25*). Research is under way to simplify this procedure and extend it to the neutral oxygenated fraction.

Linear programmed temperature gas chromatography is used in the analysis for aliphatic hydrocarbons. Separations are performed on 2 m columns. Where possible at least two separations are performed on each sample. *n*-Alkanes from C_{10} to C_{42} can be readily separated in about 60 to 70 minutes. Air samples contained about 3 to 7 percent *n*-alkanes in the aliphatic fraction. *n*-Alkanes from C_{17} to C_{35} were represented in this fraction.

7. *Nondestructive spectrophotofluorometric procedures.*—With present day spectrophotofluorometers, which are equipped with 2 monochromators, excitation and emission spectra of fluorescent compounds can be obtained in the 220 to 800 mμ region. Even though the intensity values are not absolute and the wavelength maxima are affected by the type of

photomultiplier tube used, a spectrophotofluorometer is extremely useful in the characterization and estimation of fluorescent compounds in mixtures. The intensity values obtained on the instrument have to be calibrated daily with a standard, usually quinine.

The excitation spectrum of a pure compound in solution, as determined by common spectrophotofluorometers in use today, is closely similar to the absorption spectrum of this same compound. Differences noted are that the excitation spectrum has less fine structure and the analogous bands of the two types of spectra are not exactly at the same wavelength. These differences, which eventually may be circumvented, are due to imperfections in the spectrophotofluorometer.

The emission spectrum of a pure compound is a crude mirror image of the absorption spectrum shifted to longer wavelengths. In the spectra of the majority of fluorescent organic compounds, the long wavelength band of the absorption spectrum is at shorter wavelength than the short wavelength band of the emission spectrum.

The most intense excitation spectrum of a fluorescent compound is obtained at the most intense emission wavelength maximum of the compound, and vice versa. The same excitation spectrum should be obtained at each emission wavelength maximum, and vice versa. If the spectra obtained under these conditions are different, then fluorescent impurities are present.

Another factor of importance in analytical work is that the relative intensities of excitation and emission wavelength maxima of fluorescent compounds can be varied over a wide range, so that the intensity at some particular excitation or emission wavelength may be hundreds of times greater for one particular compound than for another (*23*). For some compounds the relative intensities can be varied over 1000-fold. In the same manner relatively weak fluorescing compounds can be made relatively strong at their most intense excitation and emission wavelength maxima.

On the basis of all these factors, it is possible in many instances to obtain the pure emission spectrum of an aromatic hydrocarbon in a mixture by determining the emission spectrum of the mixture at an appropriate excitation wavelength maximum of the aromatic hydrocarbon. In addition, the pure excitation spectrum of the aromatic hydrocarbon in a mixture sometimes can be obtained at the appropriate emission wavelength maximum of the hydrocarbon. With these techniques it has been possible to detect benzo[*a*]pyrene, naphtho[2,3,*a*]pyrene, perylene, or anthanthrene in artificial mixtures containing 25 to 50 polynuclear aromatic hydrocarbons (*23*). For many polycyclic hydrocarbons, fluorescence spectral techniques give greater selectivity and sensitivity than comparable absorption spectral methods. The high sensitivity possible with the spectrophotofluorometer is demonstrated by the detection of 30 pg (30×10^{-12} g) of anthanthrene in pentane solution.

Uncorrected excitation and emission spectra of a large number of polycyclic hydrocarbons have been reported (*23*). With these data and the use of previously discussed techniques, polynuclear aromatic hydrocarbons in airborne particulates have been characterized (*13*, *18*). Quenching effects due to high concentrations of the compound being analyzed or of the background material have to be taken into account, however, since the shape of the emission spectrum of a compound can be radically altered by the quenching effect.

A spectrophotofluorometric method for the estimation of benzo[*a*]pyrene in the neutral aromatic fraction of airborne particulates has been described (*18*). When attempts were made to apply this method to the benzene-soluble fraction of airborne particulates, the wavelength maxima used in the analysis were found to have shifted and extremely high values, apparently caused by interferences, were obtained for benzo[*a*]pyrene. As applied to the benzene-soluble fraction, however, the procedure gives values that might be indicative of the extent of aromatic compound pollution in the air.

Increased selectivity, sensitivity, resolution, and reproducibility can be expected of future spectrophotofluorometers.

RESULTS OF ANALYSIS

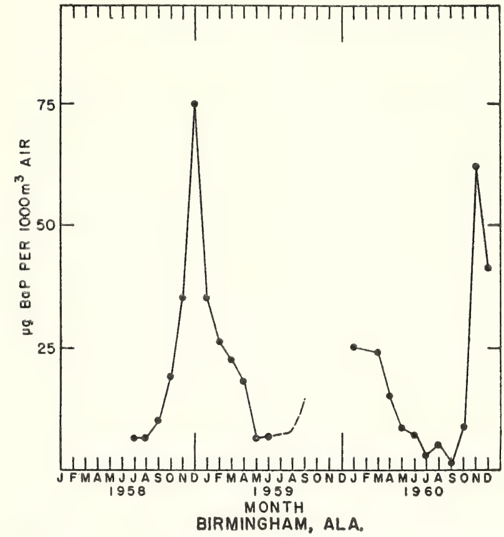
Preliminary studies have been made of 1) atmospheric pollution by benzo[*a*]pyrene, 2) pollution by other polycyclic aromatic hydrocarbons, 3) variations in the concentrations of hydrocarbons in various types of air pollution, and 4) variations in the concentrations of hydrocarbons in diverse sources of atmospheric pollution. Some results of these preliminary studies are summarized in the following paragraphs:

Atmospheric Pollution by Benzo[*a*]pyrene

An examination of the air of more than 130 urban and nonurban areas in all sections of the country has disclosed that benzo[*a*]pyrene is universally present (*19*). Samples from urban sites gave higher values than those from nonurban sites. The highest urban and nonurban values were obtained in Eastern areas of the country. The concentrations in urban and nonurban sites all over the country varied from 0.01 to 75 μg per 1000 m^3 of air. In the airborne particulates the percentage of benzo[*a*]pyrene varied from 0.00001 to 0.041; in the benzene-soluble fractions of airborne particulates the percentage varied from 0.0009 to 0.3.

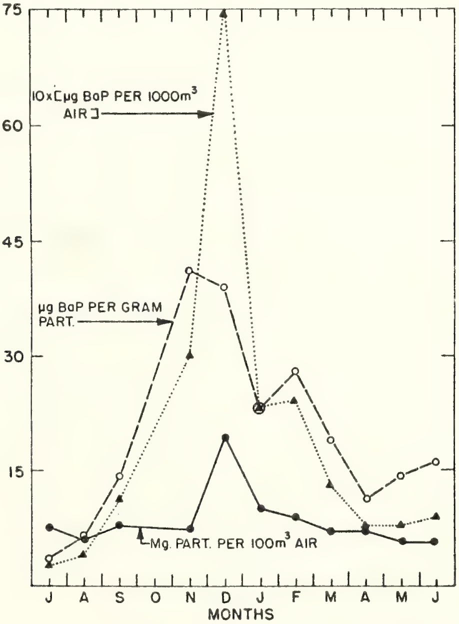
The seasonal variation of benzo[*a*]pyrene has been studied for a number of cities. In Birmingham the records of seasonal variation over several years show that benzo[*a*]pyrene concentrations are higher in the winter months than in summer months (text-fig. 1). This is true of practically all cities studied.

Text-figures 2 and 3 show that the increase in benzo[*a*]pyrene concentration during the winter months is not due to an increased amount of particulates but to an increased amount of benzo[*a*]pyrene in the particu-

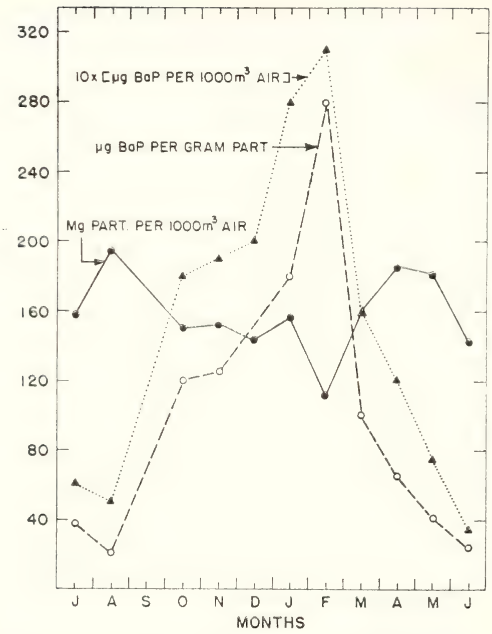


Text-figure 1.—Monthly concentration of benzo[*a*]pyrene in μg per 1000 m³ of air for Birmingham, Alabama, over 3 years.

lates. In one community the largest monthly concentration of benzo[*a*]pyrene in the air was found when the particulate concentration was at a minimum (text-fig. 3).



Text-figure 2.—One-year study of the monthly change in the concentrations of benzo[*a*]pyrene per 1000 m³ of air, benzo[*a*]pyrene per g of particulates, and airborne particulates per 100 m³ of air for San Francisco.



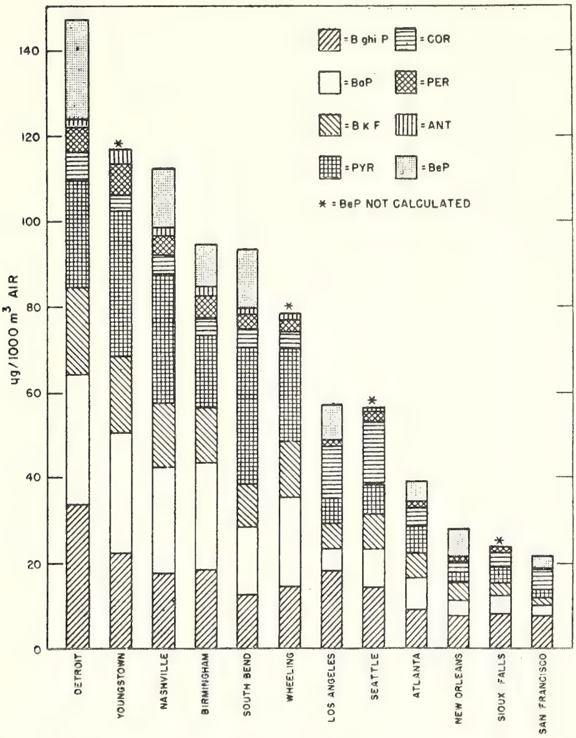
Text-figure 3.—One-year study of the monthly change in the concentrations of benzo[*a*]pyrene per 1000 m^3 of air, benzo[*a*]pyrene per g of particulates, and airborne particulates per 1000 m^3 of air for Detroit.

Atmospheric Pollution by Polynuclear Aromatic Hydrocarbons

Benzo[*ghi*]perylene was the major polynuclear hydrocarbon in airborne particulate samples collected in the summers of 1958, 1959, and 1960 at Atlanta, Birmingham, Cincinnati, Detroit, Los Angeles, Nashville, New Orleans, Philadelphia, San Francisco, and South Charleston. For these samples the amount of benzo[*a*]pyrene relative to the amount of benzo[*ghi*]perylene (the BaP/BghiP ratio) ranged from 0.2 for Los Angeles to 0.8 for Birmingham. Very low ratios appear to be characteristic of auto exhaust pollution. In most winter samples benzo[*ghi*]perylene was usually present in greater concentration than benzo[*a*]pyrene, except in communities where coal was a winter fuel, in which case benzo[*a*]pyrene values were higher; examples of this were found in data from Birmingham, Nashville, South Bend, Wheeling, and Youngstown. The BaP/BghiP ratio for the 1958 winter samples ranged from 1.3 for Youngstown to 1.5 for Nashville (*20*). Similar ratios were found in 1960 samples. High ratios seem to be typical of coal combustion pollution.

Comparisons of polycyclic hydrocarbons found in the air in various communities are shown in text-figure 4 for winter samples and text-figure 5 for summer samples. The very strong seasonal changes in the concentrations of various hydrocarbons can be seen.

In a continuous study of the concentrations of benzo[*a*]pyrene, perylene, and anthanthrene in the air, benzo[*a*]pyrene always has been found in



Text-figure 4.—Comparisons of concentrations of various polynuclear aromatic hydrocarbons in diverse urban atmospheres. Winter, 1959.

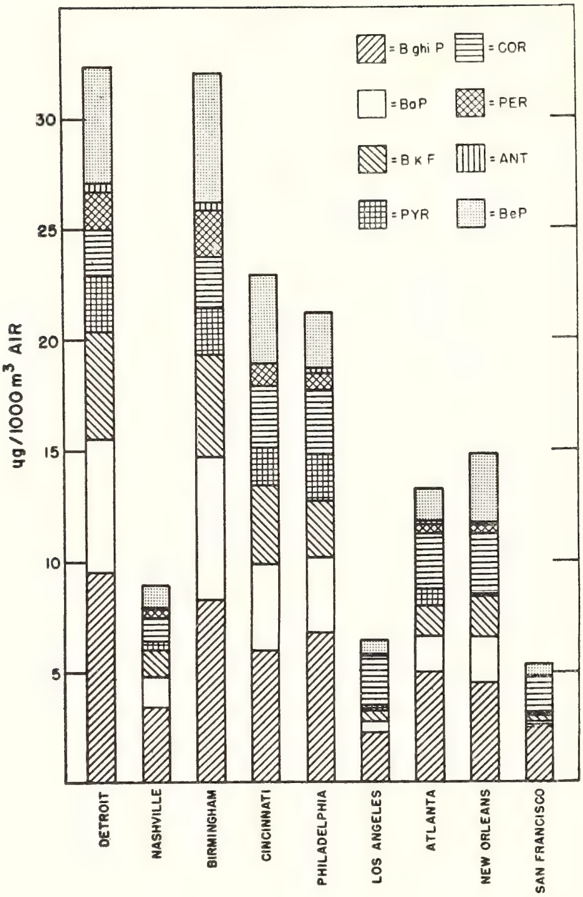
much higher concentrations than the other two, whereas anthanthrene usually is present in the lowest concentrations. This relationship holds all year round (text-fig. 6).

A comparison of urban and nonurban atmospheric samples with source samples showed that the relative proportions of pyrene as compared to the other hydrocarbons is low for both urban and nonurban samples. The previously discussed studies of 1958 to 1960 disclosed that the pyrene to benzo[*a*]pyrene ratio (P/BaP) varies usually from about 0.1 to 3, with the vast majority of values below 1. In source samples and probably in recently polluted air samples the values usually would be well over 1. The concentration of pyrene in urban air usually decreases in the summer, but so does the P/BaP ratio (text-fig. 7). It would seem that pyrene is less stable in the atmosphere and more volatile from particulates than benzo[*a*]pyrene, and, since laboratory experiments indicate this also (*26*), this ratio may serve as an indication of the "age" of the pollution.

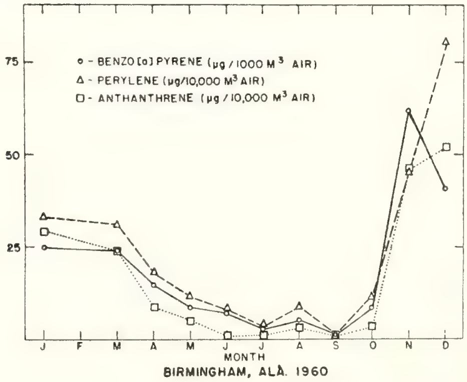
The many types of hydrocarbons in atmospheric and source samples have been listed (*20*).

Hydrocarbon Concentrations in Various Types of Air Pollution

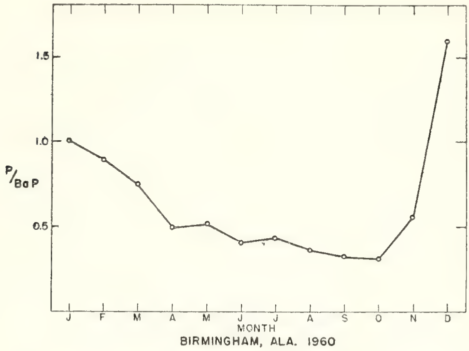
Auto exhaust.—Benzo[*a*]pyrene concentrations in airborne particulates collected from indoor auto-inspection safety lanes, enclosed urban parking



Text-figure 5.—Comparison of concentrations of various polynuclear aromatic hydrocarbons in diverse urban atmospheres. Summer, 1958.



Text-figure 6.—One-year study of the monthly change in the concentrations of benzo[*a*]pyrene, perylene, and anthanthrene.



TEXT-FIGURE 7.—One-year study of the monthly change in the P/BaP ratio.

garages, and Los Angeles downtown areas vary widely (*19, 20*). In particulate samples believed to be derived from auto exhaust fumes, the benzo[*a*]pyrene to coronene (BaP/Cor) ratio was usually less than 1, whereas the BaP/BghiP ratio was usually less than 0.6 (*20*). In all cities for which studies have been made thus far these two ratios decrease in the summer months. Since these ratios are also low in auto exhaust particulates, it would seem that auto exhaust pollution contributes a proportionally greater amount to the total pollution during the summer months. Supporting these results is the report of increased amounts of benzo[*ghi*]perylene and coronene in the particulates obtained from auto tunnels (*27*).

A preliminary analysis of the intake and exhaust air from an auto-tunnel ventilation system showed that the air in the tunnel (*28*) is polluted by a high concentration of organic compounds (table 1). The increase in benzene-soluble material in the airborne particulates obtained from the tunnel is about 7½ times as great as the increase in particulates. The concentration of benzene-soluble fraction of particulates in the tunnel air was 45 times that in outside air, and that of benzo[*a*]pyrene 265 times. The concentration of benzo[*a*]pyrene in the tunnel air is extremely high compared with that in American urban and nonurban atmospheres. The concentration of aliphatic compounds in auto tunnel air should also be extremely high. The composition of these tunnel samples, in terms of additional polycyclic aromatic hydrocarbons, high-molecular-weight

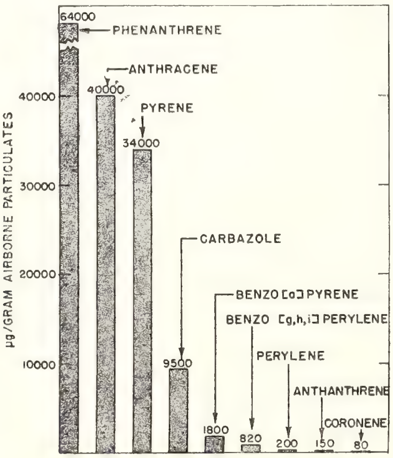
TABLE 1.—Air pollution by hydrocarbons in an auto tunnel in μg per 1000 m^3 of air

Material	Incoming air	Outgoing air
Particulates	102, 000	609, 000
Benzene-soluble materials	5, 350	241, 000
Benzo[*a*]pyrene	2. 6	690
Benzo[*ghi*]perylene	4. 7	990
Coronene	1. 4	530
BaP/BghiP (ratio)	0. 6	0. 7
BaP/Cor (ratio)	1. 9	1. 3
Benzene-soluble material in particulates, percent	5. 3	40

aliphatic hydrocarbons, and other compounds, is being studied more thoroughly.

Coal combustion pollution.—It has been shown that polycyclic hydrocarbon concentrations in the benzene-soluble fraction of airborne particulates and in the air usually are high in urban and nonurban areas near the coal-mining belt of the East (*20*). Examination of the winter airborne particulates from this area disclosed that the BaP/BghiP and BaP/Cor ratios usually are above 1. Use of these ratios is one means of differentiating this type of pollution as opposed to auto exhaust. The differences can be very large; for example the BaP/Cor ratio in downtown Los Angeles was 0.2 in July, 1959 (0.5 in February), whereas that in downtown Youngstown averaged 8 in January, February, and March, 1959.

Coal-tar pitch pollution.—Urban air polluted with coal-tar pitch fumes has been examined also (*29*). This type of pollution is characterized by large amounts of anthracene, phenanthrene, and pyrene. Thus far, only in coal-tar pitch pollution has carbazole been found in readily determinable amounts. The very high concentrations of various hydrocarbons are shown in table 2. Anthracene and phenanthrene are too volatile to be retained on the collecting filter unless they are present in very large quantities; however, tar particles collected on the filter help to retain these volatile hydrocarbons. Pollution from coal-tar pitch fumes is characterized by much higher P/BaP, BaP/BghiP, and BaP/Cor ratios in the benzene-soluble fraction of the airborne particulates than that from coal combustion or auto exhaust. The amount of coronene found in these samples is relatively small. In some cases particulate samples obtained during a roof-tarring operation contained up to 1.9 percent benzo[*a*]pyrene; percentages of pyrene, phenanthrene, and anthracene in the airborne particulates were 28, 14, and 11, respectively. Typical analysis of a sample obtained during a sidewalk-tarring operation



Text-figure 8.—Concentrations of various hydrocarbons in an air sample polluted by coal tar pitch.

TABLE 2.—Polycyclic hydrocarbon concentrations in air samples polluted by coal tar pitch

Hydrocarbon	Near industry handling coal tar pitch		Roof-tarring operation		Sidewalk-tarring operation	
	μg/g benzene-soluble	μg/1000 m³ air	μg/g benzene-soluble	μg/1000 m³ air	μg/g benzene soluble	μg/1000 m³ air
Anthracene	>2, 500	>1. 9	>110, 000	>81, 000	>49, 000	>850, 000
Phenanthrene	>9, 700	>7. 5	>140, 000	>100, 000	>210, 000	>3, 700, 000
Pyrene	13, 000	10	280, 000	200, 000	59, 000	1, 000, 000
Benzo[*a*]pyrene	950	0. 73	19, 000	14, 000	4, 500	78, 000
Perylene	190	0. 15	4, 900	3, 500	1, 400	24, 000
Benzo[*ghi*]perylene	720	0. 55	1, 500	1, 100	2, 300	40, 000
Anthanthrene	140	0. 11	158	112	a*	a
Coronene	67	0. 05	a	a	a	a
Carbazole	2, 500	1. 9	81, 000	57, 000	36, 000	620, 000
P/BaP ratio	14		14		13	
BaP/BghiP ratio	1. 3		13		2	
BaP/Cor ratio	15		a		a	

*Not analyzed.

is shown in text-figure 8. From some of the latter air samples, as much as 78 mg of benzo[*a*]pyrene, 850 mg of anthracene, 1 g of pyrene, and 3.7 g of phenanthrene in 1000 m^3 of air have been obtained.

Because of these extremely high concentrations of polycyclic hydrocarbons caution must be exercised by people working in or near hot coal tar pitch. It has been reported that coal-tar pitch fumes, which attack the skin and eyes, are a greater hazard to people than asphalt fumes (*30*). The reaction is definitely more significant on sunny days because of the photosensitizing effect of sunlight. Acute skin erythema after exposure to the sun has been reported in "anthracene" workers (*31*).

Hydrocarbon Concentrations in Sources of Atmospheric Pollution

Coal tar pitch.—Except for coal tar pitch, the chemical composition of most sources of air pollution in terms of the higher boiling components is essentially unknown. Analysis of coal tar pitch has shown that it contains large amounts of many types of polycyclic hydrocarbons (*32–34*). Analysis of a sample that was used in tarring roofs gave the following percentages of hydrocarbons; phenanthrene, 4.8; benzo[*a*]pyrene, 3.4; benzo[*e*]pyrene, 1.8; benz[*a*]anthracene, 1.4; anthracene, 1.4; benzo[*ghi*]perylene, 1.3; pyrene, 1.0; chrysene, 0.9; fluoranthene, 0.34; anthanthrene, 0.3; perylene, 0.25; and coronene, 0.1. The percentages fluctuate fairly widely, depending on the history of the pitch sample.

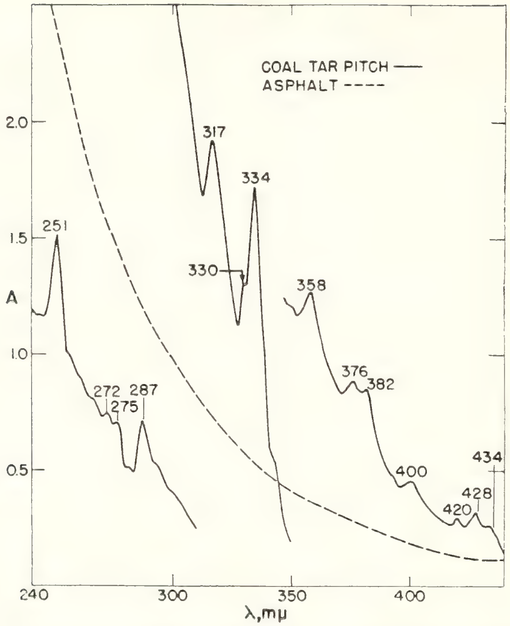
Examination of approximately 24 samples of coal tar pitch and asphalt from various sections of the country disclosed that these two tars can be readily differentiated by a comparison of the ultraviolet-visible absorption spectrum of their pentane extracts (text-fig. 9). The presence of pyrene, perylene, anthanthrene, etc., is indicated by the appropriate bands. Some of these compounds can be estimated without preliminary separation.

Auto exhaust.—The hydrocarbons found in auto exhaust particulates have been tabulated (*20*). Auto exhaust particulates collected from the exhaust pipe of an automobile operated under controlled conditions on a chassis dynamometer (*35*) have been analyzed for hydrocarbons by the column chromatographic—absorption spectral procedure. The sample from the tailpipe was passed through a series of bubblers and traps maintained at reduced temperatures, then through a glass-fiber filter. Results of 5 tests under varying operating conditions are listed in table 3.

The P/BaP ratio in all the auto exhaust samples was very much higher than that in the atmosphere, even in communities where auto exhaust fumes are considered a major pollutant, *e.g.*, Los Angeles, which has values usually below 1. The possible role of collection losses and atmospheric decomposition resulting in this substantial difference in pyrene content needs to be determined. The BaP/BghiP ratios are about as low as those found in urban atmospheres where auto exhaust fumes are a major pollutant. For example, the values in Los Angeles range around 0.3; in table 3 they average 0.3.

Table 3.—Concentration of hydrocarbons in auto exhaust fumes

Test	Benzene-soluble fraction (g/1000 m³ gas)	μg/1000 m³ Gas			Ratios	
		Pyrene	Benzo[*a*]pyrene	Benzo[*ghi*]perylene	P/BaP	BaP/BghiP
I	93	100, 000	2, 600	6, 200	39	0. 42
II	310	280, 000	7, 400	17, 000	38	0. 44
III	320	123, 000	2, 500	8, 600	48	0. 30
IV	400	260, 000	4, 400	11, 000	59	0. 40
V	1, 250	790, 000	12, 000	50, 000	66	0. 44



Text-figure 9.—Ultraviolet-visible absorption spectra of pentane solutions of coal tar pitch and asphalt.

A more comprehensive study of the composition of auto exhaust fumes is under way.

Incinerator effluents.—The percent of benzo[*a*]pyrene in particulates from the stacks of well-run incinerators usually is less than 0.00001. This is actually a lower concentration than that found in nonurban airborne particulates in which percentages of benzo[*a*]pyrene range from about 0.00001 to 0.005 (*20*).

A comparison was made of the composition of airborne particulates collected from an incinerator before and after scrubbing (table 4): The nonscrubbed particulate sample was collected by passing some of the effluent from the furnace (before it was washed with water) through a series of bubblers and traps maintained at reduced temperatures and then through a glass-fiber filter. The scrubbed sample was collected direct from the stack (*35*). In both sets of samples the aliphatic fraction was almost negligible. In the sample obtained before scrubbing, the following hydrocarbons were found: phenanthrene, pyrene, fluoranthene, benzo[*ghi*]fluoranthene, chrysene, benzo[*a*]anthracene, naphtho[2,1,8,7-*klmn*]xanthene, benzo[*a*]naphthacene, benzo[*a*]pyrene, benzo[*k*]fluoranthene, benzo[*ghi*]perylene, and coronene. In addition sulfur and many other unidentified polycyclic compounds were present. In the samples obtained after scrubbing, evidence for the presence of most of these compounds could not be found; the amounts of the compounds present were considerably less than those found in samples taken before scrubbing (table 4). The greatest decrease, however, was in the concentration of the polycyclic hydrocarbons after scrubbing (table 4).

TABLE 4.—Effect of scrubbing on incinerator effluent

Hydrocarbon	Before scrubbing (μg/g) Benzene-soluble	Before scrubbing (μg/g) Particulates	After scrubbing (μg/g) Benzene-soluble	After scrubbing (μg/g) Particulates
Pyrene	17, 000	27	360	1. 3
Fluoranthene	15, 000	23	550	2. 0
Benzo[*e*]pyrene	3, 900	6	97	0. 4
Benzo[*a*]pyrene	2, 000	3	15	0. 06
Benzo[*ghi*]perylene	11, 000	18	110	0. 4
Coronene	4, 900	8	110	0. 4

A more thorough study of the composition of incinerator effluents is under way. The composition of various types of source effluents polluting the air warrants further investigation.

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DISCUSSION

DR. HOFFMANN: Dr. Sawicki, we have tried to compare the amounts of benzo[*a*]pyrene inhaled by a heavy smoker and a person living on 42d Street, New York. We have reached conclusions different from yours. With Dr. Lyons, we have also tried to compare various polynuclear aromatic hydrocarbons obtained from cigarette smoke and from the particulate phase of auto exhaust in the same way. We have done this only on the basis of biological testing. I think it is not absolutely proper. Maybe Dr. Falk can give a better comment to compare the amount in the air, since some of the particulate phases are collected in the hair of the nose and so on. Studies by Dworki and Wynder have shown that a heavy smoker who inhales deeply inhales 70 percent of the particulate phase, and with this the polynuclear hydrocarbons. I think the respiration of persons in the city is quite different. We do not inhale deeply, and some of the particulate phase is caught in the nose. So I think we should be cautious when comparing cigarette-smoke condensate and air pollutant.

CHAIRMAN SAWICKI: We have pointed out in our paper that these comparisons are crude, especially in the case of cigarette smoking, where many times most of the cigarette burns away without being puffed—whenever the smoker's attention wanders. The values we have reported we considered as crude maximal values, especially for cigarette smokers.

DR. FALK: In inhalation we find that the particles from the atmosphere almost completely enter the lung, contrary to your suggestion that much of the particulate is retained in the nose. However, the other point is that after these particles get in, they are immediately removed from the lung. A completely normal individual in an atmosphere of soot does not retain the soot in his lungs. It is not that the soot did not get there, but it was immediately gotten rid of. We found the only way that we could alter this wonderful defense mechanism on the basis of ciliary action was to inhibit ciliary action. We did this by irritating lung tissue with cigarette smoke or Los Angeles smog. In this way a tremendous amount of soot was retained in the lung. So there are other factors involved besides the nose that have an effect on the retention of carcinogenic material by lung tissue.

Also, the compound mentioned previously, 3*H*-benzo[*b*]carbazole, is quite carcinogenic. This may explain why chrysene in the hands of some investigators is carcinogenic and in the hands of others is not.

CHAIRMAN SAWICKI: One further point: Cigarette smoke contains small amounts of polynuclear aromatic hydrocarbons in the particulate phase compared to the amount in urban airborne particulates. However, we must use caution in making this comparison, for although the hydrocarbon carcinogens are such a small fraction of cigarette tobacco tar there is a tremendous number of other types of chemicals with unknown physiological properties. Consequently, cigarette smoke or airborne particulates may contain something else that is even more potent.

DR. KATZ: I noticed in your data that in one set of figures the City of Detroit is outstanding for its content of total polynuclear hydrocarbons, which probably include a considerable amount of carcinogens. We have been studying the Detroit-Windsor problem for a number of years, and we were always struck by the fact that from the standpoint of pollution by particulate matter Windsor-Detroit is a highly polluted area. You can always collect substantial quantities of dust in the air, much more than from Los Angeles air. What in your opinion is the predominant reason for this high total of polycyclics in relation to the dust in the Detroit area?

CHAIRMAN SAWICKI: This increased amount of polycyclic hydrocarbons could be due to a relatively larger amount of coal-combustion pollution coupled with fewer sunnier days. However, we would have to do a thorough study of sources polluting the Detroit area to prove this conjecture. In some areas we get even higher values than that.

DR. PAYNE: We have been talking here about aromatic hydrocarbons. I would like to emphasize also that we should not forget compounds other than the hydrocarbons. Baltimore, for example, has a very high chromium content in the air. I think we should broaden our outlook toward things other than the organics.

MRS. HOFFMANN: Dr. Sawicki, you have pointed out the difficulties in obtaining average samples of air pollution. I should like to ask how you try to standardize for wind speed and wind direction in an average sample. In our analysis of samples collected with the truck sampling unit, we have found a 30 percent variation in benzo[*a*]pyrene even when the samples were collected at the same spot at almost the same time. Have you any data on the effect of wind direction on the collection of samples?

CHAIRMAN SAWICKI: I would like to refer this question to Mr. Stern.

MR. STERN: The sampling procedures involve an apparatus in which a filter is placed in a horizontal position with a shelter over it, so that the air intake is around all 4 sides. The effect of wind is the same because the air has to pass under a barrier and up and then down to get to the filter. This serves the dual purpose of knocking out the very heavy particles that cannot make the downward-upward transition. Therefore, we do not get very large particles, which would normally be considered those that would fall out onto the ground, but we get suspended particles. The sampling apparatus is not responsive to wind direction in that it takes the sample from whatever direction the wind may be coming.

MR. FOX: (Frank Fox, U.S. Public Health Service, Cincinnati.) As a small adjunct to the work in polycyclics here in the Center, we have done some preliminary work on gas chromatography of the long-chain paraffin hydrocarbons. A few of the chromatograms we obtained recently were shown. Most of the work in the literature on paraffins in waxes has been in large proportion in the C-31 area. Because of the difficulty of obtaining some calibration compounds of the longer chains, we are presently limited to C-32. Only recently we obtained some C-36 paraffin. We tried quite a variety of substrates and found that silicone grease and the methyl silicone polymer SE-30 yield quite clean separations in relatively short periods. We obtained a chromatogram of an aliphatic fraction from the benzene extract of air particulate matter. This work is in a preliminary stage, and I cannot say which of the bands are derived from normal and which from branched alkanes. However, the rather equal spacing of the chromatographic peaks suggests that these are normal paraffins. As Dr. Lyons mentioned, there is a technique of forming urea adducts to effect some separation of branched and normal paraffins. We shall most likely employ this technique to clean up the benzene extract prior to gas chromatography. In addition, we intend to use, and have used to some extent, chromatography on an alumina column with rather low amounts of water in the alumina—about 12 percent by weight. We have one other technique for cleaning up the normal paraffins and that is to employ a prechromatographic column using dual columns, a precolumn of molecular sieves that would hold back these normal paraffins. We then back-flush at high temperature. Some recent literature has reported the possibility of flushing these normal paraffins off the molecular sieve. These early data are quite encouraging. We have managed to obtain peaks separated by about 1 inch.

MR. GAGER: At the recent gas chromatography symposium in Lansing, Dr. Widom from Shell in England reported the use of high-temperature molecular sieves, 3A, I believe. He reported higher efficiency in separation of the normals from the isoparaffins in the range of the C-24. I suspect you are referring to his report.

Quantitative Analysis of Benzo[*a*]pyrene in Vapors Coming From Melted Tar [1]

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SUMMARY

The atmosphere in the vicinity of workmen handling molten coal tar has been found to contain high concentrations of benzo[*a*]pyrene. Samples were collected by means of an electroprecipitator. The approximate amounts of benzo[*a*]pyrene inhaled by workmen in the various operations were calculated. In some cases approximately 320 μg was inhaled within an hour.—NCI Monogr. No. 9: 221–223, 1962.

TO SUPPLY information regarding a case of lung cancer to an expert chosen by the court of justice of insurance companies, we were asked to perform analyses for benzo[*a*]pyrene in vapors coming from molten coal tar. This study was limited to the hazards incurred in certain professions and its purpose was to determine the amount of benzo[*a*]pyrene inhaled by a workman handling molten coal tar.

In the tarring process, the workmen melt tar in a large container and stir it continually. The melted tar is then transferred to a small container and carried to the workmen's place of work. There they mix the melted tar with cork powder, and then spread the hot mixture on the walls to be treated. These workmen inhale vapors from the tar in considerable amounts when they stand close to the large container, when the melted tar is being transferred, and when they mix it with the cork powder. Very often this work is performed under poor conditions, *e.g.*, in basements with poor ventilation.

Results of Quantitative Analysis of Benzo[*a*]pyrene

The first step in our study was to calculate the temperatures of the melted tar. In contrast to previous estimates, the temperatures were higher: 220° to 310° C in the large stock container, and 180° to 210° C in the small containers used to carry the material.

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[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

On the basis of these findings, we heated in our laboratory masses of 1 kg of tar at 200° and 300° C, and we collected vapors at a distance of 20 cm from the surface of the tar samples. Analysis proved that the vapor pressure of benzo[*a*]pyrene increases rapidly with temperature. At 300° C, the vapors are richer in benzo[*a*]pyrene than the original melted tar:

The solid tar contains 3.0 percent benzo[*a*]pyrene.

The vapors produced at 200° C contain 0.5 and 0.6 percent benzo[*a*]-pyrene.

The vapors produced at 300° C contain 4.3 and 4.4 percent benzo[*a*]-pyrene.

Such data were not sufficient for us to calculate the doses inhaled by a workman. Therefore, we collected new material, not in the laboratory but at the place where the work was performed.

1) Vapors were collected at about 40 cm above the large container with the stock of melted tar. When the temperature of the tar was 310° C, the vapors contained 1.4 percent benzo[*a*]pyrene; when 270° C, the vapors contained 0.8 percent benzo[*a*]pyrene.

2) Material was then collected close to the workman while he was mixing the tar with cork powder and applying the tar to various surfaces. When the temperature of the tar was 210° C, the vapors contained 0.5 percent benzo[*a*]pyrene.

3) Material was also collected close to the workman while he leveled the surface. In this case, the airborne particulates contained 0.2 percent benzo[*a*]pyrene.

The material was collected each time with an aspiration of 4250 liters per hour. The workman, when performing his work without an exceptional effort, inhales about 500 liters per hour. Therefore, because we knew the weight of material collected by aspiration during 1 hour, we were able to calculate without difficulty the amount of benzo[*a*]pyrene contained in 500 liters:

1) At a distance of 40 cm from the large stock container, the following amounts were collected within 1 hour: at 310° C, 1.78 g of material containing 25.3 mg of benzo[*a*]pyrene; at 270° C, 0.89 g of material containing 6.9 mg of benzo[*a*]pyrene. Thus, within 1 hour at the large stock container, a workman would inhale 2.98 mg of benzo[*a*]pyrene when the temperature of the tar is 310° C, and 0.81 mg when the temperature is 270° C.

2) Where the workman was manipulating the tar at a temperature of 210° C, 0.42 g of material containing 2.2 mg of benzo[*a*]pyrene was collected in 1 hour. Thus, during this process within 1 hour a workman would inhale 0.26 mg of benzo[*a*]pyrene.

3) Where the workman was leveling the surfaces of tar at a temperature of about 20° C, 0.21 g of material containing 2.7 mg of benzo[*a*]pyrene was collected in 1 hour. Thus, at this site within 1 hour a workman would inhale 0.32 mg of benzo[*a*]pyrene.

These data were used as a basis for calculating the average period of time of each operation involved in this work. We were then able to calculate the amount of benzo[*a*]pyrene inhaled by a workman during a day of work.

Obviously, it is not possible to determine the exact amount of benzo[*a*]pyrene deposited in the lungs, because the classification of the various diameters of particles, as well as the number of particles per unit of volume, have not yet been studied.

METHODS FOR COLLECTION AND ANALYSIS

Collecting Vapors

Vapors were collected by means of an electroprecipitator. Voltage at the electrodes was 12,000 volts, and the air volume sampled was 4250 liters per hour. For each collection operation the exact time of functioning of the turbine is noted.

Contents of the electrodes were dissolved in benzene. The solvent was evaporated warm under a current of nitrogen. During this operation, the weight of collected material was determined.

Analysis of Benzo[*a*]pyrene

Analysis was easy, since the amount of benzo[*a*]pyrene was relatively high.

Fifty mg of tar vapors was weighed, and the separation by chromatography was done at once. A column, 11 mm in diameter, was packed to a height of 50 cm with the standardized alumina of Riedel de Haen A.G., Seelze-Hannover, Germany. The column was eluted at normal pressure with successive 100 ml volumes of C. P. Merck cyclohexane containing 1 and 2 percent of absolute ethanol, respectively, and then with approximately 1200 ml of cyclohexane containing 3 percent absolute ethanol.

Polycyclic hydrocarbons were eluted as follows: anthracene, pyrene, chrysene, and finally, at the end of the elution of chrysene, the benzo[*a*]pyrene, together with a brown-colored zone. All the eluates containing benzo[*a*]pyrene were gathered. The quantitative analysis was made on an aliquot sample, occasionally diluted by cyclohexane, to which had been added surcharges of a solution of benzo[*a*]pyrene of known concentration.

The analysis of the collected material was considered accurate within 5 percent.

Interim Report on the Study of Techniques for Determination of Polycyclic Aromatic Hydrocarbons in Air [1]

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SUMMARY

The described tests showed that some procedures involved in the determination of polycyclic aromatic hydrocarbons in air can be subject to error. The tests showed that samples collected over long periods, particularly at elevated temperatures, are subject to a reduction in their hydrocarbon content. Even samples kept in the laboratory for periods as short as 3 weeks can be altered; however, cyclohexane extracts of smoke samples are quite stable. Other tests showed that fluorescent polycyclic aromatic hydrocarbons were not detected in the vapor phase in town air. Spectrophotometric procedures in which a narrow baseline is used were more reliable than those in which wider baselines were used. All these tests showed that great care must be exercised in the determination of polycyclic aromatic hydrocarbons in air. These results represent an interim report on these reliability tests; results of further tests will be published elsewhere.—NCI Monogr. No. 9: 225–233, 1962.

THE POLLUTION of air by combustion products has long been a source of annoyance and has now become, with the increase of industrial activity and great growth of towns, a serious health hazard as well. In the tarry material present in polluted air, polycyclic aromatic hydrocarbons can be found, among which is benzo[*a*]pyrene, a notable carcinogen. Since 1946 when Hieger (*1*) first identified benzo[*a*]pyrene, a number of methods have been developed for the determination of polycyclic aromatic hydrocarbons (*2–9*). The tarry material in town air is a very complex mixture of substances, and to identify and determine polycyclic aromatic hydrocarbons, specially sensitive techniques have had to be devised. A number of the early methods have now been improved (*5–9*) but investigation of the sampling, extraction, and spectrophotometric procedures used in the determination of polycyclic hydrocarbons has been given little attention. This paper gives an account of a number of tests applied to assess the reliability of these procedures.

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[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

[2] Thanks are due Dr. P. J. Lawther, Director of the Medical Research Council Air Pollution Research Unit, and Mr. Waller, also of the Unit, for help in the planning of some of these tests. Thanks are also due Mrs. J. Dale and Mrs. R. Hogan for technical assistance.

SAMPLING PROCEDURES

In the past very little experimental work to assess the efficiency and reliability of sampling equipment used for the collection of air solids, to determine polycyclic aromatic hydrocarbons, was carried out. The following is a list of the tests we have applied:

1) Determination of the losses and collection efficiency of polycyclic aromatic hydrocarbons at room and somewhat elevated temperatures
2) Determination of stability of samples after collection
3) Measurement of the losses of pure benzo[*a*]pyrene and pyrene, when air is drawn through filter papers on which they are collected
4) Determination of the polycyclic aromatic hydrocarbon vapor content of the air

Determination of Losses and Collection Efficiency of Polycyclic Aromatic Hydrocarbons at Room and Somewhat Elevated Temperatures

It has been shown (*10*) that pure benzo[*a*]pyrene is appreciably volatile at temperatures above 100° C, and to determine the possible losses of hydrocarbons during their collection at room and somewhat elevated temperatures, clean air was drawn through a portion of a collected sample.

Test with a large-volume sampler (*Staplex sampler*).—Two identical samples of smoke were collected from polluted air at 1.2 m^3 per minute over a period of 2 hours on 8 × 10 inch glass fiber sheets. Through one of these samples filtered air was drawn for 2 hours, again at 1.2 m^3 per minute. The air was filtered by a fresh piece of glass fiber sheet placed in front of the collected sample, the air being drawn through both sheets. After this experiment, the treated and untreated sheets were analyzed and the results compared (table 1).

TABLE 1.—Comparison between two samples, one of which was subjected to filtered air

Hydrocarbon	Concentration of hydrocarbons μg per g smoke	
	(A) Sample	(B) Sample with extra air
Fluoranthene	1, 310	1, 240
Benzo[*e*]pyrene	228	268
Pyrene	1, 016	953
Coronene	107	111
Anthracene	190	244
Benzo[*ghi*]perylene	387	368
Benzo[*a*]pyrene	572	608
Anthanthrene	140	133
Total	3, 950	3, 925

From the table it can be seen that the total amounts of 8 hydrocarbons collected did not change significantly when extra air was drawn through the filter paper on which the smoke had been collected. The concentrations of individual hydrocarbons show some variation which could, however, be within the limits of experimental error.

The hydrocarbons determined seem therefore not to be destroyed or volatilized significantly by the atmosphere in which they exist when sampled with a Staplex sampler.

Test with a small-volume sampler.—A sample of particulate pollutants collected from the air inside a gas works over a period of 5 hours on an 8 × 10 inch glass fiber sheet with a large-volume sampler was cut into portions and treated in several different ways. Three portions were treated as follows:

(*a*) One was analyzed immediately.

(*b*) One was left exposed to the warm gasworks for 3 weeks.

(*c*) Another portion was analyzed after being kept in the dark for 3 weeks. Some of the cut portions were placed in 2 inch filter holders and treated with filtered air at low rates of flow as follows:

(*d*) The warm (up to 50° C) filtered air of the gasworks was passed through at a rate of 3 liters per minute for 3 weeks.

(*e*) Warm filtered laboratory air at 50° C was passed through at 3 liters per minute for 3 weeks.

(*f*) Filtered laboratory air at room temperature was passed through at 3 liters per minute for 3 weeks.

Results of the analyses of these samples are given in table 2.

TABLE 2.—Results of analysis of samples subjected to various treatments

Hydrocarbon	Concentration of hydrocarbon μg per 100 m^3 air					
	No treatment, immediate analysis	Analysis after 3 weeks	Room temperature laboratory air drawn through for 3 weeks	Hot laboratory air at 50° C drawn through for 3 weeks	Sample exposed to hot gasworks air for 3 weeks	Hot gasworks air (up to 50° C) drawn through for 3 weeks
Fluoranthene	30. 9	19. 5	7. 4	7. 2	44. 8	10. 2
Phenanthrene	14. 0	5. 9	4. 1	2. 9	8. 0	3. 4
Benzo[*e*]pyrene	5. 8	7. 1	3. 9	5. 6	5. 1	6. 6
Pyrene	23. 7	15. 6	3. 2	3. 4	50. 6	8. 2
Benzo[*ghi*]perylene	6. 6	6. 0	6. 3	5. 5	7. 1	4. 9
Benzo[*a*]pyrene	20. 0	19. 1	19. 0	16. 6	18. 3	14. 0
Anthanthrene	2. 3	2. 5	1. 6	2. 0	2. 1	1. 2

Table 2 shows that some of the lower-molecular-weight hydrocarbons were lost when the sample was kept in the laboratory for 3 weeks, the loss being more pronounced among the lower hydrocarbons. If hot (up to 50° C) gasworks air or laboratory air at approximately the same temperature was drawn through a portion of the sample, then the loss was similar to that when air at room temperature was used. A sample placed in a hot gasworks atmosphere showed losses in the concentrations of a few hydrocarbons but increases for fluoranthene and pyrene. The reason for the increases is not fully understood, but it could possibly be due to the condensation on the filter of tar droplets from the gasworks air.

The results indicated that samples collected on filter papers over several weeks would be affected by the air drawn through the filter and would become poorer in hydrocarbon content. The effect of increasing the air temperature from room temperature to 50° C did not seem to increase the loss very markedly.

It would seem that the results of analyses of samples collected by low-rate pumps over periods of a few weeks were not reliable. Losses were more marked with the lower hydrocarbons, but samples collected over longer periods may have suffered a very serious loss of the higher hydrocarbons. These experiments were performed on gasworks samples as part of a survey of retort house atmospheres. (Similar experiments with a high-volume sampler gave results in accord with those quoted in table 1.) We have no reason to doubt that results of the low-flow-rate experiments described can be applied to urban pollution.

Stability of Stored Samples

Falk *et al.* (*11*) showed that pure hydrocarbons are not stable in air. To test the stability of hydrocarbons in smoke samples collected from air, a sample of urban air solids collected with a high-volume sampler was analyzed immediately after collection and after storage of the sample for 1 year. The year-old sample had been kept in a sealed envelope in the dark. The results are given in table 3.

TABLE 3.—Comparison of hydrocarbon content of air solids before and after storage for 1 year

	Concentration of hydrocarbons μg per g smoke		
Hydrocarbon	Analyzed immediately after collection	Analyzed 1 year after collection	Loss (%)
Fluoranthene	225	18	92
Pyrene	328	38	88
Benzo[*a*]pyrene	111	76	32
Benzo[*e*]pyrene	71	55	23
Anthanthrene	70	55	21
Benzo[*ghi*]perylene	252	226	10
Coronene	142	140	1

From table 3 it can be seen that the loss of some of the hydrocarbons is very high indeed and appears to invalidate the use of results obtained from analyses made some time after the collection of these samples. The results of an analysis made immediately after the collection and another analysis of the same material made only 3 weeks later with a fresh extract are shown in table 2. These results again show considerable losses for the lower-molecular-weight hydrocarbons, but losses for the higher-molecular-weight hydrocarbons were insignificant.

Losses of Pure Hydrocarbons When Air Is Drawn Through Filters

Known amounts of pure hydrocarbons were placed on the surface of 8 × 10 inch glass fiber sheets by uniform application of an acetone solution of the hydrocarbon. When the solvent had evaporated, filtered air was drawn through the sheets. After the sheets were exposed to air, they were extracted with cyclohexane and the amount of hydrocarbon remaining was determined spectrophotometrically.

The higher-molecular-weight hydrocarbons are the least affected by this treatment. When 500 μg of benzo[*a*]pyrene was placed on a filter paper and air at room temperature drawn through at 1.5 m^3 per minute for 2 hours, none was lost; whereas, after the same treatment, 35 percent of a similar weight of pyrene was lost. The actual weight of material on the filters is important, since if only 50 μg of pyrene is treated with air for 2 hours, only 3 percent is recovered. Ketcham and Norton (*12*) found a 25 percent loss of benzo[*a*]pyrene when 20 μg was placed on paper sheets and air was drawn through for 3 days.

Examination of the spectrum of the extracted hydrocarbon showed changes which could be accounted for by the presence of oxidation products. It is not certain whether the loss was due entirely to oxidation, since some evaporation of the hydrocarbons may also have occurred.

Attempt to Collect Polycyclic Hydrocarbon Vapor From Air

An attempt to demonstrate fluorescent polycyclic aromatic hydrocarbons in the vapor phase in town air was made by drawing filtered air through a drechsel bottle containing pure liquid paraffin. This solvent in which benzo[*a*]pyrene and many other hydrocarbons fluoresce very strongly was used because the volatility of the more commonly used solvents made them unsuitable for use in a long experiment. The liquid paraffin was not fluorescent at the beginning of the experiment; when 81.6 m^3 of air was aspirated through it over a period of 38 days, no fluorescence of the paraffin was detected after this period, and thus it can be concluded that no measurable amounts of fluorescent hydrocarbons exist as vapors in the air at atmospheric temperatures.

Discussion

The tests have shown that the time of sampling is important. Large-volume samplers with glass fiber filters for collection of samples over short periods have been the most reliable. When longer sampling periods (several weeks) are required, the low-rate samplers are necessary and some loss of hydrocarbons would occur during collection.

Air passed at high rates at room temperature through pure hydrocarbons placed on filters causes the loss of some of the hydrocarbons; however, so far no such loss has been detected in samples of smoke collected from the air at these flow rates, and it would seem likely that the carbon or tarry particles must in some way protect the hydrocarbons during sampling. This protective effect may be caused in part by adsorption of the hydrocarbons on carbon, which can be made to adsorb hydrocarbons strongly (*13*).

Samples should be analyzed as soon as possible after collection, because some of the hydrocarbons are lost during a delay of even a few weeks.

Filtration appears to be a highly efficient means of collecting polycyclic aromatic hydrocarbons from the particulate matter in the air. It is almost certain that some hydrocarbon vapor is emitted from combustion processes, but carbon particles are simultaneously formed, and it is very likely that the vapors condense and will be readily adsorbed by these particles, rendering the hydrocarbons nonvolatile.

EXTRACTION

The polycyclic aromatic hydrocarbons in collected air solids can be extracted readily with cyclohexane. Great care must be exercised when these extracts are reduced to small volume, since some of the lower-molecular-weight hydrocarbons are appreciably volatile. Generally, it is found adequate to reduce the extract from 300 ml to about 50 ml by distilling off the solvent and finally reducing the volume to very small bulk (2 ml) by evaporation at room temperature with an electric blower.

Pure hydrocarbons dissolved in cyclohexane and other solvents are not very stable (*14*). Extracts of smoke-containing hydrocarbons are generally colored (yellow, green, brown, or red), however, and the hydrocarbons present are almost completely stable for several months. Since hydrocarbons in solution can be destroyed by ultraviolet light (*14*), it is presumably the strongly light-absorbing properties of the extracts which prevent such decomposition.

An example comparing analyses of an extract of a fresh smoke sample and the same extract after it had been kept in the dark for 6 months is shown in table 4.

TABLE 4.—Comparison of analyses of a cyclohexane extract of smoke analyzed immediately and 6 months later

	Concentration of hydrocarbons in μg per g	
Hydrocarbon	Extract analyzed immediately	Extract analyzed 6 months later
Fluoranthene	465	475
Phenanthrene	243	202
Benzo[*e*]pyrene	83	74
Pyrene	394	392
Coronene	64	56
Anthracene	55	46
Benzo[*ghi*]perylene	149	149
Benzo[*a*]pyrene	146	158

The results of the analyses of the extract show that the results obtained are essentially the same even after 6 months' storage.

Since smoke samples are not very stable over long periods and cyclohexane extracts of the hydrocarbons are stable, samples that cannot be analyzed immediately ought to be extracted and analyzed later. When a measurement of an average annual concentration of hydrocarbons is required, monthly smoke samples can be extracted immediately after collection to reduce the loss of hydrocarbons before analysis of the pooled extracts. For convenience, the cyclohexane extract of the previous month can be used as the extracting liquid for the following month and so on throughout the year. (Repeated boiling under reflux of the cyclohexane extracts of smoke samples did not cause any loss of hydrocarbons.)

ABSORPTION SPECTROPHOTOMETRY

A number of techniques involving the examination of absorption spectra have been used to determine the amounts of hydrocarbons present in chromatographic fractions. Baseline techniques (*3*, *6*, *7*) have been the most reliable for the quantitative determination of hydrocarbons.

The selection of the wavelengths on either side of the peak is important. Cooper used wide baselines (up to 20 mμ) for the measurement of hydrocarbons. Extensive experience of many analyses of polycyclic aromatic hydrocarbons has shown that errors can be caused by the use of wide baselines; these are due to spectral interference by other hydrocarbons and some unidentified compounds. To reduce the effect of the interference, baselines between 5 and 8 mμ have been adopted.

The actual extent of the errors involved when narrow and wide baselines are used in the determination of hydrocarbons was measured experimentally. In this test pyrene was determined in a solution with and without oil as a contaminant by use of both baseline procedures. Table 5 shows the comparison between the optical heights obtained when narrow and wide baselines were used.

TABLE 5.—Comparison between errors caused by wide and narrow baselines

Hydrocarbon	Contaminant	Wavelengths used (mμ)		Slit width (mm)	Optical heights of hydrocarbons determined: In pure cyclohexane (A)	Optical heights of hydrocarbons determined: In cyclohexane-oil mixture (B)	Ratio B/A (%)
Anthracene	Lubricating oil	366. 0 376. 0 386. 0	Wide	0. 06	0. 1500	0. 1160	77
		372. 0 376. 0 380. 0	Narrow	0. 06	0. 1155	0. 1090	94
Pyrene	Gas oil	327. 0 335. 0 343. 0	Wide	0. 30	0. 3395	0. 2835	84
		332. 0 335. 0 338. 0	Narrow	0. 30	0. 2225	0. 2135	96

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DISCUSSION

MR. BEGEMAN: I wonder if Dr. Commins has made a similar correlation in his traffic studies as shown for carbon monoxide, lead, and coronene that correlates benzo[*a*]pyrene with traffic density?

DR. COMMINS: I showed the correlation with benzo[*a*]pyrene, but had difficulty in interpreting these results since in London we have a very special, narrow, two-lane tunnel that has some bends. Large vehicles hold up the cars behind them. So while cars are coming around the corner in one direction, there is a queue of vehicles waiting in the other direction. We think that the Blackwall tunnel is not suitable for the study of the effects of the emission of benzo[*a*]pyrene from particular vehicles. Other tunnels, like the one under the Mersey in Liverpool, would be more ideal in this respect. Perhaps some of your tunnels in New York could be used.

CHAIRMAN SAWICKI: We have investigated the effect of air passing through the airborne particulate sample on the filter. Four pairs of samples collected during January were studied. Approximately 3500 m^3 of air was passed through the glass fiber filter to collect each sample. Following this, about the same volume of filtered air was pulled through half the samples; thus, duplicates were collected at the same time and place. The only difference was that, for each pair of samples, one had twice as much air passed through as the other. In these duplicate samples we found no definite differences in particulate weight, in the weight of benzene-solubles, or in the concentrations of benzo[*a*]pyrene, benzo[*ghi*]perylene, or coronene.

DR. COMMINS: Have you investigated the low-molecular-weight hydrocarbons, as for example pyrene?

CHAIRMAN SAWICKI: We did see some small changes in pyrene, while in anthracene and phenanthrene we saw very large changes.

Some Observations on the Relative Quantities of Air Pollutants in Various Locations [1]

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SUMMARY

Information about the amounts and distribution of some pollutants in air is very precisely known. Other pollutants have not been so fully studied, and others are not completely established. Further, the total amounts and relative proportions are subject to well-understood variation according to season, climate, and time of day. Superimposed upon these variations are changes due to development of new areas, to the imposition of legal restraints, and to the use of new materials. In these circumstances of over-all flux it is unwise and unfair to concentrate too closely on any single constituent of air pollution or on any group of constituents. In England the major constituents of total air solids are not organic. Among the organic portion of total air solids the known carcinogenic compounds are present in very minor amounts. It thus appears useful to review quantitatively all the known air constituents with the object of reassessing carcinogenic risks. One might then discover whether new research is needed to obtain a more exact estimate of risk and determine more accurately the role of various air pollutants in carcinogenesis—NCI Monogr. No. 9: 235–250, 1962.

THIS PAPER deals with analytical results rather than with methods. The few new methods that are described are thus considered to be of secondary importance and are described in the appendixes. The objects of the paper are to review the proportions of pollutants in air in relation to as many variables as possible and to assess the relative importance of the known constituents in carcinogenesis under various conditions. No single constituent can reasonably be neglected as a factor in carcinogenesis until it has been proved to be without influence.

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[1] Presented at a Symposium on The Analysis of Carcinogenic Air Pollutants, Cincinnati, Ohio, August 29–31, 1961.

[2] I wish to thank a number of my former and present collaborators for their contributions to the material presented in this paper: Dr. R. L. Cooper who developed methods for polycyclic aromatic hydrocarbons; Dr. J. R. Stanbury who applied these and other methods to the study of total air solids and commenced the studies presented in this paper; Mr. and Mrs. Alfredo Ferreira for examining numerous ash samples by emission spectroscopy, and Mr. I. E. Burrows who is studying air solids with me now.

I should like to record my thanks also to the late Professor Sir Ernest Kennaway whose valuable, inspiring encouragement was always so freely given. I have also had most useful discussions with Dr. P. Lawther, Director of the Medical Research Council's Air Pollution Unit, and his colleagues Mr. R. E. Waller and Dr. B. T. Commins.

At the outset, it is important to place limits upon the study and these have been provided in part by the organizers of the symposium. As analysts, we participators are obviously required to look for compounds known to be carcinogenic, to consider qualitative and quantitative factors, and attempt to assess these factors. As analysts too, we also wish to account for every constituent and to list suspected substances of unknown biological activity. This is a very heavy task, especially since conventional investigation of air pollution is directed to quite different ends. It is difficult because of the extraordinary varying systems we have to study; and the difficulties are enhanced by the chemical lability of the systems.

At the end of the study, which includes some newly reported sampling and analytical procedures, it is hoped that a useful reassessment of the role of air pollutants may be achieved and the relative importance of various analytical surveys may be defined. Certain gaps in knowledge are revealed in the study and in consequence new methods may be developed, which will give rise to a new type of survey that should lead to more true estimates of risk.

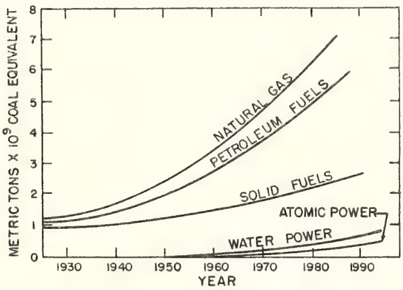
EXISTING SAMPLING AND ANALYTICAL METHODS

In this section, discussions are confined to methods in current use for surveys in Great Britain. These analytical methods and reporting systems are not directed toward an estimate of carcinogenic risk, and it would therefore be surprising if they could afford more than the slightest help toward the present study. In fact, they provide more help than might at first be thought possible, and so an examination of air pollution methods and results has been made to assess their usefulness in this field and to attempt to find gaps in the knowledge they present. This type of assessment has been applied rather incompletely, but has yielded interesting results.

If we accept the thesis that the main burden of general air pollution is due to combustion, we come to the inevitable conclusion that it is bound to increase as time passes because of the increase in the power and heating needs of the world. Text-figure 1 shows the rate of growth of power utilization in the noncommunist world (figures are not available for the communist areas), and the rates are extrapolated into the future. Unless some entirely new methods of pollution control are evolved, the total amount of general pollution due to combustion is bound to increase, even if the carbon content can be reduced by more efficient combustion.

Most regular analytical studies are concerned with one or a few of the many polluting constituents. A partial examination of this kind can give only partial information, and so it is most important to attempt, at least occasionally, analyses as complete as possible on polluted air. This we have commenced at Sir John Cass College by the study of solids collected from air filtration plants, and some preliminary results are presented in this paper and compared with other analytical figures.

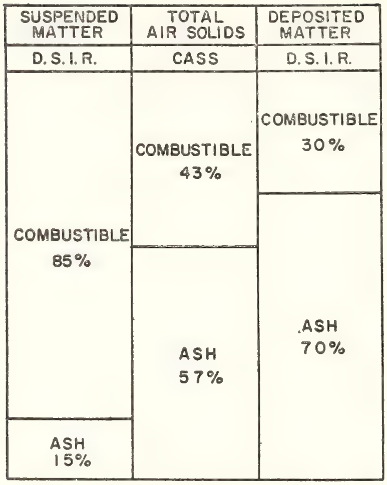
When one examines the status of knowledge on air pollution, two grave deficiencies are at once apparent: incompleteness of detailed long-term surveys and the lack of analytical information, even of qualitative information, in both long- and short-term studies. These points are illustrated by information taken from surveys made in England. Since 1914, a nation-wide network of observing stations has been developed to measure the amount of general air pollution by recording the blackness of particulate material filtered from the air and equating this to "suspended matter." At the same time "deposited matter" has been measured with precipitated water. Sulfate, chloride, ammonia, calcium content, and acidity of the water have been determined and recorded regularly. In the insoluble matter, ash, combustible matter and "tar" (combustible matter soluble in carbon disulfide) have been measured also. Sulfur dioxide amounts have also been recorded. At first sight this appears to be an impressive achievement, but its deficiencies become apparent upon closer examination. Text-figure 2 shows the average amounts of ash and combustible matter in total air solids collected from an air filtration plant. It also shows the corresponding amounts for the arbitrary subdivisions of "deposited matter" and "suspended matter" or "smoke," and as would be expected, the composition of the total solids is between these extremes.



TEXT-FIGURE 1.—The rate of growth of power utilization in the noncommunist world.

ANALYSIS OF TOTAL AIR SOLIDS

The high air velocities in filtration plants are sufficient to trap most of the finest particles by impact. The combustible matter is mostly carbon, but it contains volatile constituents as well. The portion extractable with carbon disulfide and called "tar" was not examined until recently. Indeed, Professor Clemo in 1953, in his Presidential address to the Chemistry Section of the British Association for the Advancement of Science, said (with almost complete truth), "It was a surprise to learn recently that no single organic constituent of the smoke scourge has been isolated and definitely identified, although for too many years past this evil has been stressed." Professor Clemo was not entirely correct, for we had at this

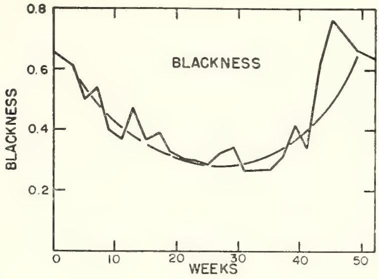


Text-figure 2.—Average amounts of ash and combustible matter in total air solids.

time commenced work on the isolation of polycyclic aromatic hydrocarbons from combustion products and air pollutants. The combustible matter contains a very large number of constituents, and so does the ash.

The quantitative analytical results so far presented in this paper are averaged for a year in one place. Well-known cycles of quantities are found, however, if shorter periods are studied. The yearly cycle has a maximum for air pollutants in winter and a minimum in summer, the weekly cycle shows lower smoke pollution at weekends in both summer and winter, and the daily cycle shows maxima at stoking and fire-lighting times (morning and evening). The yearly cycles are especially interesting and lead to the presentation of new results on total air solids collected at approximately 2-week intervals throughout the year 1955 from an air-purifying plant near Liverpool.

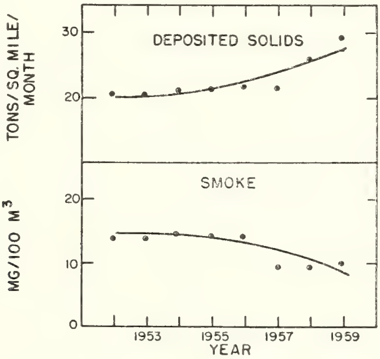
The total air solids collected in an air filtration plant include both particle-size subdivisions defined as "suspended matter" and "deposited matter." Such samples have the merit that they can be large and thus furnish plenty of material for analysis. A number of investigations have been made on the material collected in this way, and the first of these to report on is the blackness of the total air solids. The reflectance of light from a well-pressed-down flat surface of the bulk air solids was measured, and the reciprocal of this arbitrary measurement (standardized on powdered charcoal) was termed the blackness. Text-figure 3 shows the change of blackness of air solids throughout the year. It demonstrates the inaccuracy of the method employed throughout the official United Kingdom measurements for "suspended matter" determinations, since the calibration (first made in 1917 in London and still used) assumes a constant blackness for air solids regardless of time and place. According to Waller, 1961, determinations of suspended matter by gravimetry may vary by as much as 2.5 times from determinations made by reflectometry.



Text-figure 3.—Change of blackness of air solids throughout the year.

(Gravimetric analysis, or at least regular gravimetric standardization of the blackness method, is strongly recommended here.)

Additional support is given for the universal use of a gravimetric measurement by studies made in the City of London by the methods of the Department of Scientific and Industrial Research before and since it became a smokeless zone. The blackness of London air solids is decreasing; therefore, determinations by examination of stains on filter papers indicate less smoke pollution. But the total burden of air solids is rising, which shows that ash and other light-colored solid pollutants are increasing. Text-figure 4 shows the changes with time in the city of "smoke" (suspended matter) and total deposited matter.

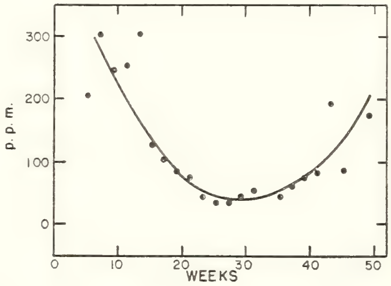


Text-figure 4.—Changes with time in the city of suspended matter and total deposited matter.

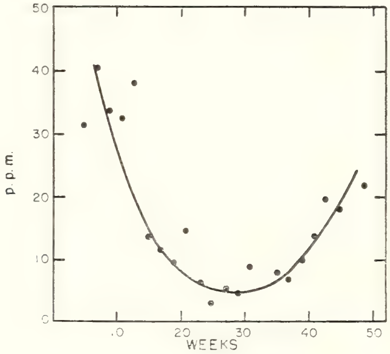
Because combustion processes are the major source of air pollution, the present tendency to improve efficiency of combustion appliances and create smokeless zones in large cities is not necessarily reducing the total burden of air pollution; it is mainly changing its color in England. But, if it can be proved that the powerful organic carcinogens associated with partial combustion are effective, the smaller amounts of these formed during more efficient combustion may be a double advantage.

Polycylic aromatic hydrocarbons have been demonstrated in most partial combustion products and have been determined in polluted air in many locations.

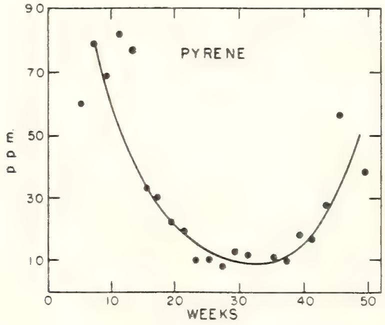
Eight of these compounds were determined by us in the total solids filtered from Liverpool air throughout the year 1955, and text-figure 5 shows the changes with time for all these compounds. Among these compounds are the potent carcinogen benzo[*a*]pyrene and others of less powerful nature, which have been determined regularly in air in a number of laboratories since 1951. Text-figure 6 shows the changes of benzo[*a*]pyrene amounts with time, and text-figures 7 and 8 show changes in amounts of pyrene and fluoranthene, respectively, with time.



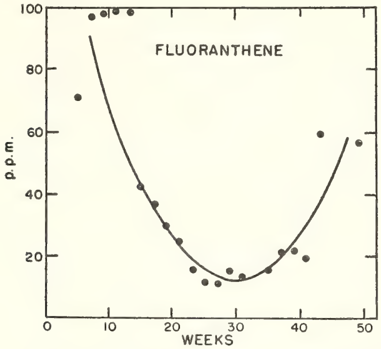
TEXT-FIGURE 5.—Change in concentration with time of 8 polycyclic aromatic hydrocarbons in total solids.



TEXT-FIGURE 6.—Changes in the amount of benzo[*a*]pyrene with time.



TEXT-FIGURE 7.—Changes in the amount of pyrene with time.



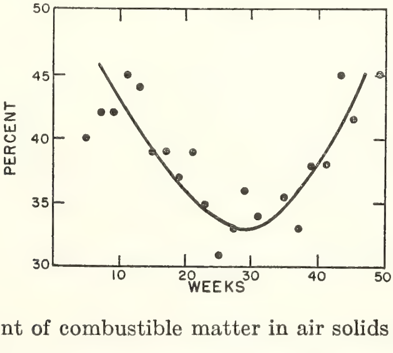
TEXT-FIGURE 8.—Changes in the amount of fluoranthene with time.

When the total aromatic hydrocarbons and benzo[*a*]pyrene are considered, it is important to note that small proportions only of these constituents are present in relatively large amounts of other organic compounds. Less than 0.03 percent of the organic constituents of air solids is benzo[*a*]pyrene.

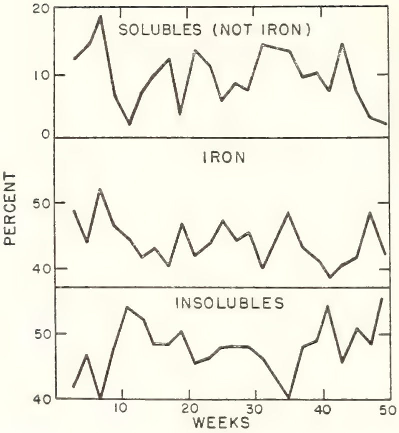
Possibly too much emphasis has been placed on the results of analyses of the polycyclic aromatic hydrocarbons, but until the other organic constituents have been identified and tested for carcinogenic activity, this point will remain undecided.

The fluctuation of amounts over yearly cycles for all the constituents so far quantitatively determined and presented are of the same type. Indeed, they represent a series of related factors; all could be described as indexes of the quantity of partial combustion products in the air in which they were determined, and if the locality and type of combustion appliances responsible for the pollution are known, they are plainly a measure of the amount of inefficient combustion there. This final point is supported by study of the amounts of combustible matter in air solids plotted against time in text-figure 9. This curve is of the same general shape as all the preceding.

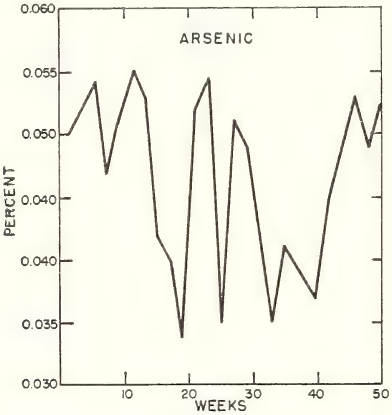
Other analytical investigations were made on the ash of specimens from the same collection, and of these many were found in proportions which varied randomly throughout the year. Thus, iron, arsenic, ger-



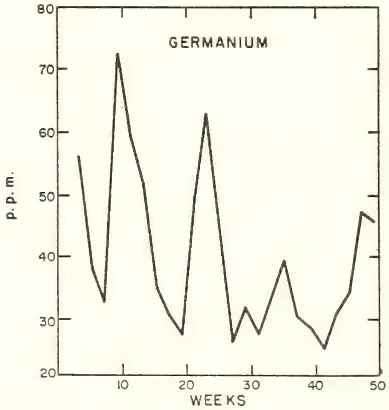
TEXT-FIGURE 9.—Amount of combustible matter in air solids plotted against time.



Text-figure 10.—Iron in air solids plotted against time.

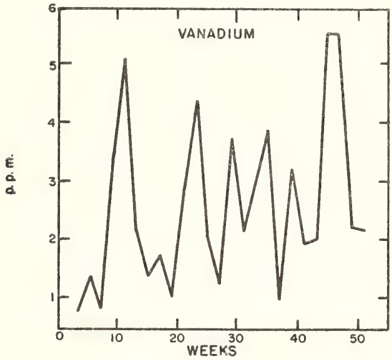


Text-figure 11.—Arsenic in air solids plotted against time.

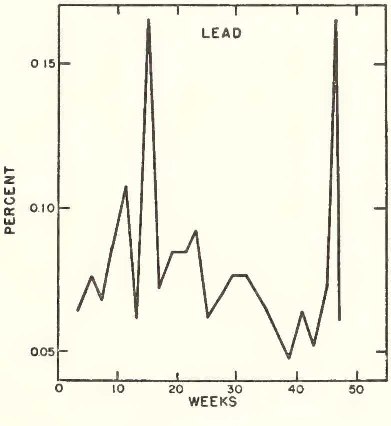


Text-figure 12.—Germanium in air solids plotted against time.

manium, vanadium, and lead were all determined and the amounts are shown graphically in text-figures 10, 11, 12, 13, and 14. There is no annual cyclic regularity in these pollutants, and, from such irregular results, the conclusions can have no more significance than that the ash-producing fuels are somewhat variable in nature and therefore probably variable in origin. This is, of course, true!



TEXT-FIGURE 13.—Vanadium in air solids plotted against time.

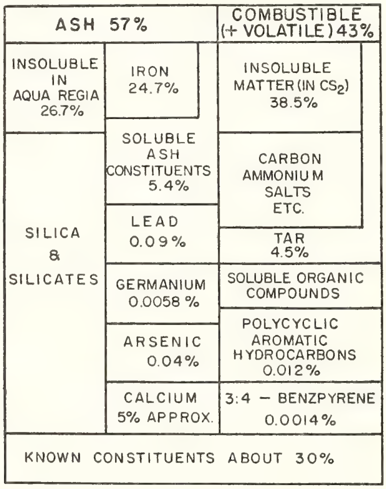


TEXT-FIGURE 14.—Lead in air solids plotted against time.

It is important that average amounts of a number of elements in the ash of air solids are now known and if the effort were to be made still more could be determined. The number of elements we detected by use of spectroscopy is 23 to date, and these are listed in Appendix 2. Although these constituents are not within those specified as being appropriate to the present symposium, it may well be that some have a role in carcinogenesis.

Of the list of known constituents in air pollution, some have carcinogenic activity when appropriately tested. The significance of such lists is not easy to assign, since so little is as yet known on the mechanism of carcinogenesis because of the many complicating factors such as promoter action and anticarcinogenic factors or inhibitors.

To review the present position, text-figure 15 shows the state of present quantitative knowledge on total air solids. Only about 30 percent of the total air solids in samples have been analyzed quantitatively, and there is, therefore, much scope for further investigations.


ASH 57%
COMBUSTIBLE (+ VOLATILE) 43%
INSOLUBLE IN AQUA REGIA 26.7%
IRON 24.7%
INSOLUBLE MATTER (IN CS2) 38.5%
SOLUBLE ASH CONSTITUENTS 5.4%
CARBON AMMONIUM SALTS ETC.
SILICA & SILICATES
LEAD 0.09%
TAR 4.5%
GERMANIUM 0.0058 %
SOLUBLE ORGANIC COMPOUNDS
ARSENIC 0.04%
POLYCYCLIC AROMATIC HYDROCARBONS 0.012%
CALCIUM 5% APPROX.
3:4 – BENZPYRENE 0.0014%
KNOWN CONSTITUENTS ABOUT 30%

Text-figure 15.—The state of present quantitative knowledge on total air solids.

APPENDIX 1. SUMMARIES OF ANALYTICAL METHODS FOR TOTAL AIR SOLIDS

1. *Blackness determination.*—This was a measurement in arbitrary units. The method needs development if it is to be used in standardized determinations on a routine basis. The specimen was filled into a rectangular metal tray and smoothed with a plate of glass to give an even surface. It was then placed in a simple photoelectric reflectometer in which a parallel beam of light from a tungsten filament lamp was allowed to fall on the surface, and the reflected light was measured by means of a galvanometer and photoelectric cell. The reciprocals of the readings were plotted as arbitrary measurements of blackness and the constancy of performance was insured by use of a specimen of powdered charcoal as a standard.

2. *Polycyclic aromatic hydrocarbons.*—A sample of about 0.5 g was exhaustively extracted with 10 ml of cyclohexane, and the resulting solution was reduced to 2 ml by distillation. The reduced solution was transferred to a chromatographic column of partly active alumina and eluted with cyclohexane. About 50 fractions of less than 3 ml were collected; each was made up to 3 ml and its ultraviolet spectrum plotted between 200 and 450 mμ. The hydrocarbons present were recognized and then determined by peak height measurement (*10*, *27*, *52*, *54*, and *55*).

3. *Arsenic.*—A sample of about 0.05 g was oxidized by digestion with 5 ml concentrated sulfuric acid containing a trace of cupric sulfate. When all the carbon had been removed 10 ml of water was added, followed by 20 ml of 20 percent hydrochloric acid and enough stannous chloride solution to complete the color change. In all-glass apparatus 15 ml was distilled, and to the distillate 40 ml of water and 5 ml of stannated hydrochloric acid were added. This solution was tested in a standard Gutzeit apparatus (British Pharmacopœia) with 10 g of zinc. The resulting stain was compared with standard colors to estimate the amount of arsenic present.

4. *Iron.*—Preliminary experiments showed that after attack with aqua regia, all the iron present was in the solution, though some insoluble matter containing silica remained. The air solids were ashed to constant weight by heating in a platinum crucible in a muffle furnace at 600° C. A sample of 0.04 g was attacked by heating with 4 ml of hydrochloric acid and 1 ml of nitric acid. The liquid was diluted and centrifuged, and the solution poured into a standard flask. The residue was washed twice with water and then centrifuged. The washings were added to the flask and made up to 100 ml. An aliquot of this solution was taken; its color absorbance was determined with a Unicam SP500 spectrophotometer after addition of potassium thiocyanate solution. The iron percentage was calculated from a calibration curve prepared by use of a standard solution of ferric alum.

5. *Germanium.*—The ash from 1 g of air solids was mixed intimately with 1.5 g of sodium carbonate in a platinum crucible, covered with a 0.5 g of sodium carbonate, heated with a Meker burner until the temperature approached 1000° C, and held at this temperature for 15 minutes. The cooled melt was warmed with 5 ml of water and heated on a hot plate till it disintegrated. Part of the melt was transferred to a 100 ml distillation flask. The remainder was transferred to a flask with the acid of 10 ml of dilute 1:1 hydrochloric acid. About 10 ml of water and 25 ml of concentrated hydrochloric acid were then added, and the whole was gently boiled for about 5 minutes, after which 20 ml were distilled and made up to 25 ml. An aliquot of this solution (10 ml) was then submitted to the phenylfluorene colorimetric method for germanium as development by Cluley (Analyst, 1951, *76*, 523) (*52*).

6. *Lead and vanadium.*—These elements were determined by the well-known internal standard method of emission spectroscopy and by measurement of the line intensities. Hilger medium and large quartz spectrometers with Hilger microphotometers were employed.

Spectroscopically pure cupric oxide was mixed with lead to give mixtures containing 2, 1, 0.5, 0.1, 0.05, and 0.01 percent lead; a mixture of the same cupric oxide with 10 percent of the ash was also prepared. The various mixtures were arced and the photographic plates examined photometrically.

A similar method was used for vanadium.

APPENDIX 2. LIST OF ELEMENTS DETECTED IN ASH FROM TOWN AIR SOLIDS

Aluminum
Arsenic
Barium
Beryllium
Boron
Calcium
Copper
Gallium
Germanium
Iron
Lead
Lithium
Magnesium
Molybdenum
Potassium
Silicon
Silver
Sodium
Strontium
Tin
Titanium
Vanadium
Zinc

BIBLIOGRAPHY ON RESEARCH INTO COMBUSTION CHEMISTRY, TOBACCO SMOKE, AND ATMOSPHERIC POLLUTION

Research into the chemistry of polycyclic aromatic hydrocarbons has been in progress for many years in the Department of Chemistry, Sir John Cass College, London. Under the direction of the former Head, the late Dr. E. de Barry Barnett, a vigorous school of synthetic organic chemistry studied this class of compound, concentrating mainly upon anthracene and its derivatives. Prominent among these workers were J. W. Cook, N. F. Goodway, C. L. Hewett, M. A. Matthews, and J. L. Wiltshire. Later Cook, with Hewett and I. Heiger, working at the Research Institute of the Cancer Hospital under the direction of Professor Sir Ernest Kennaway, isolated benzo[*a*]pyrene from a strongly carcinogenic fraction of coal tar pitch and confirmed its identity by synthesis.

At the Sir John Cass College, after World War II, the present Head of Department and his collaborators commenced a series of analytical studies aimed at the routine quantitative analysis of combustion and pyrolytic products. In 1950 the use of alumina column chromatography and ultraviolet spectrophotometry was developed for the detection and determination of polycyclic aromatic hydrocarbons by R. L. Cooper, a research student in the Department, and applied by him and a number of other research students to the examination of smokes, soots, air solids, gasworks effluents, and other products of partial combustion. The widespread presence of these hydrocarbons and the carcinogenic nature of several of them has made this research of considerable interest, and they have been supported by the Medical Research Council and the Department of Scientific and Industrial Research. During the last decade, Miss E. J. Bailey, I. E. Burrows, J. M. Campbell, Professor Adele Candeli, B. T. Commins, R. L. Cooper, Mr. and Mrs. Alfredo Ferreira, J. A. S. Gilbert, A. Goldup, Miss R. Ives, Miss P. Marsden, E. Pash, K. Persaud, and J. Stanbury have worked, under the direction of the Head of Department, upon various aspects of the composition of smokes. This work has been coordinated with similar and related projects at St. Bartholomew's Hospital, London, and at other centers of research and is still in progress.

The bibliography which follows is arranged chronologically and, so far as publications from the Department of Chemistry at Sir John Cass College are concerned, it is complete to August 1961. A number of references from other centers of research are included for especially relevant work, in which at least one author from the previously mentioned list was concerned.

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DISCUSSION

MR. STERN: I would like to comment on the idea that has been discussed that we should be looking at a lot more than just the subject of this particular symposium. I want to assure you that this is our attitude too. When we planned this conference it was not intended to circumscribe the area of our interest, but rather to focus on a particular portion of the entire problem that we know needs investigation. We fully concur that we need to put just as much effort into investigations in the gas phase, into the nonorganics, and into the organics other than the polynuclears. We are trying as hard as we can with the manpower available to fill in some of these numbers that you would like to see in literature.

DR. LINDSEY: I would like to make an addendum to my own presentation. I was very worried when we found so much iron in the Liverpool area because I thought perhaps this might be a somewhat unusual atmosphere. There is, in fact, an iron works not a long way from the antibiotic factory I mentioned. However, on examining London air we find that it contains just as much iron. There is no localized industry in London having any relationship either to iron works, hematite crushing, or mining or foundry work. So we felt comforted again.

DR. FALK: I was wondering whether in your list of inorganic constituents you were particularly fortunate in being completely free of chromium and nickel or whether you just did not feel like investigating these additional carcinogenic agents that are in existence in this country.

DR. LINDSEY: We are working on this problem.

CONCLUDING DISCUSSION

CHAIRMAN SAWICKI: The meeting is now open for general discussion.

MR. STERN: I would like to take the lead that was opened up earlier in the matter of whether we have been paying too much attention to benzo[*a*]pyrene and have reached the point where we should start looking at some other things. I can tell you that as far as we are concerned here at the Center, we got into the benzo[*a*]pyrene business because we knew nothing about it essentially so far as the distribution of polynuclears and benzo[*a*]pyrene in the air of the United States. Everywhere else in the world everybody seemed to know all about it. We were behind, and we were jealous of the knowledge of the British and the others and of how much they knew about the content of their atmosphere with regard to these organics. We had to at least catch up in our state of knowledge. I think that perhaps we have caught up with the other countries who got an earlier start. But I wonder whether this is the point where we should stop this line of investigation or whether this is an area in which we should continue. Frankly, this was one of the reasons we wanted to get everybody together here, so that we could see where we go next. If I can get a discussion started along the lines of what investigations in the next several years should be, this would be most helpful.

DR. LINDSEY: First of all I should like to dispel any ideas that I was criticizing the work going on at Taft for which I have the highest admiration. I was criticizing all of us. I feel that we may be devoting a little too much attention to polycyclics when there are so many other things that obviously need investigating because we know nothing about them. I would suggest that all of us should go on with our polycyclic work. I do not think we know nearly enough about it. I think we should study primary processes, and this we are doing in a very modest way, as for example in studies of how polycyclics are formed in flames. Here I think the excellent work of Professor Badger on pyrolysis is very important. The final point I should like to make is that since in English air we have more inorganic materials than organic and since we know less about the inorganic matter than we do about some aspects of the organic, we really ought to try to find out something of the quantitative amounts of both large and small inorganic constituents of air solids.

DR. FALK: I have come to the point where I believe that we know too much about benzo[*a*]pyrene and the other polycyclics and what we really ought to now study is the function inside the organism. As a biochemist, I am looking for the process that will finally explain why a person inhaling a certain amount of soot and also exposed to cigarettes and to rubber-tire dust in cities, as well as the other contaminants from sources of incomplete combustion, ends up not with a certain condensation of benzo[*a*]pyrene in his lungs, but with cancer of the lung. I have not spent the last 4 years emphasizing the minor ingredients in the atmosphere that may or may not be carcinogenic, like other polycyclics or epoxides or inorganic dust. I have tried rather to see whether they can get into the lung, and from the lung into the systemic circulation. I suggest that some of the host factors including pathological changes in metabolic or target sites may really ultimately turn out to be more important than whether we happen to live in Birmingham, London, or Los Angeles.

CHAIRMAN SAWICKI: Any other comments on this? Well, I disagree with Dr. Falk because I think that with all this knowledge we are supposed to have, we do not know enough about hydrocarbons in the air. We actually never analyzed for them quantitatively before, so how could we know anything about them in the air of the United States. Quantitative data on the organic composition of the air are practically nonexistent. I think all of this means we will have to work together, he in his field and the other people in their fields, put all our knowledge together, and then eventually learn more that way. I agree with Dr. Falk in one respect—I think biochemistry and physiology are very important tools in this investigation.

DR. ALTSHULLER: Well, the relevance of this I would not deny, but I would ask anyone to tell me what the concentration of epoxides is in any city in the United States or any city in the world, for example.

CHAIRMAN SAWICKI: I would like to bring up the point of what relation skin-painting experiments with animals could have to human lung carcinogenesis. Dr. Hoffmann, do you have any comments on that?

DR. HOFFMANN: No, I can only repeat that we never draw any conclusion from mouse-painting experiments to carcinogens in man. What we like to see is which factors might be involved in experimental carcinogenesis. We never draw a conclusion from this to human data. The human data can only come from epidemiological studies. I think the studies by Kotin and Falk on the inhibition of cilia movement might be a clue to the lung cancer problem. According to Auerbach, at one stage in the development of lung cancer there is an effect on the cilia of the bronchial tree, so maybe the research on the cilia of experimental animals is not so far off.

CHAIRMAN SAWICKI: Dr. Commins, after extraction with cyclohexane were there any polynuclear hydrocarbons left in the residue?

DR. COMMINS: We have studied this problem. In all the samples we examined we found only traces of hydrocarbons. Our usual procedure is to extract with cyclohexane and then follow up with an extraction of benzene. Maybe our samples are somewhat different from yours. The relative difference in amounts of other organic constituents may make the hydrocarbons more readily extractable in cyclohexane. We find it a very suitable solvent because it saves transference to a different solvent for the chromatographic and spectrophotometric procedures that follow.

DR. FALK: I have also tried to find out whether there ever is an end to an extraction of polycyclics from soot, and there is not. The compound that we were particularly bothered with is coronene because we can extract that till doomsday. At one time I did that, and I reached doomsday at close to 100 extractions, where I just had to give up because there was no end. You can see it approaching to zero, but it never reaches zero. There is always more left. This may depend on the particle size, of course. If you had large particles, maybe you would get to the end rapidly. But in this whole collection of particle sizes there is always enough of the small size present that some of the hydrocarbon is being extracted slowly. You can do that indefinitely, even with acetone. I never tried cyclohexane, but I would doubt that we can ever come to the end of all the polycyclics or even know when we should reach the end of our extraction.

DR. COMMINS: Dr. Falk, were these samples you referred to atmospheric samples? This puzzles me because I feel that air extraction procedures are reasonably reliable. I know your work where you had carbon blacks, which can adsorb hydrocarbons extremely strongly. Another question I would ask you—do you think that there is carbon in such a form in atmospheric solids that it might adsorb these components very strongly? In fact, is one of the reasons why diesel soot has a low content of hydrocarbons because some of the hydrocarbons are so strongly adsorbed that you cannot ever detect them?

DR. FALK: Yes, we have extracted carbon blacks and we have extracted stuff from the atmosphere. We did get to the point where you get so little coronene out that you feel that it does not make any difference any more. So I quite agree with you. You come to a practical end of your extraction, but you are never coming to an absolute end. The spectrum of coronene after these many extractions is still a fairly good spectrum, and there is a sizable quantity that always comes out. The particle size that we determined roughly was an average of 200 mμ for the diesel engine. There is enough of that fine particle size in there, which will probably never give up its coronene. We did an experiment in which we added some of the soot to a benzene solution containing the extract from another soot. We got rid of the extracted benzo[*a*]pyrene and other polycyclics by adsorption to the next soot. That showed us clearly that if we had originally mixed the two soots together and then tried to extract, we would have ended up with nothing. In our experiment, we could extract from one and could lose it to the other. This could only be done when we had definitive particle-size soots. These soots were usually carbon blacks, and as I was asked before, how could we get them into various particle sizes? Well, the industry does that for us. They give them away free of charge with electron-microscope pictures of the distribution. You can play as much as you want to with adsorption and dilution and mixing these particle sizes.

DR. HOFFMANN: Dr. Falk, you mentioned that you were coming closer and closer to the zero line. Is our mathematics not enough that we extrapolate and introduce a correlation factor, or would this be too dangerous?

DR. FALK: I think you could. To me it was not worth while because the quantity of coronene that was coming out could be easily covered by the general error in all our determinations. A little correction would be too small to worry about.

DR. HOFFMANN: So we are not so far off.

DR. FALK: No, I do not say that you are so far off, I just say that you can never come to an end. As long as we realize that we have not successfully completed extraction, it will be all right.

CHAIRMAN SAWICKI: Maybe Dr. Falk is trying to say that you get an 80 percent yield for the extraction over 8 hours, whatever you do. Then the rest of the 20 percent is obtained asymptotically over many years, if you want to keep on extracting.

DR. THOMAS: We did some work about 2 years ago. We did not work with atmospheric samples, but with particulate matter that we had obtained by burning under control conditions. We went to 420 extractions, and we could still keep on going.

CHAIRMAN SAWICKI: What were your conclusions?

DR. THOMAS: I think they would be similar to Dr. Falk's conclusion.

DR. HOFFMANN: Dr. Falk, I recently saw in *Tobacco Science* an article about separation of particle sizes from cigarette smoke by a centrifugation apparatus. Do you think this would be one possible way to do it? Maybe we would find their correlation. I think in studies by Kotin and Falk it was said that there is a different absorption in the lung of different particle sizes.

DR. FALK: I do not know what the instrument is that you are mentioning. I know one instrument that was made by Dr. Alexander Goetz of Cal Tech, who was actually devoted in trying to show us how to separate particles in his machine. He blows cigarette smoke into his machine, and out comes the slide or a piece of cellophane that has the particles spread along this slide, which he then looks at under the microscope. He does not see the particles; they are too small. But he sees the optical image of something that seems to be sitting on the cellophane. The difficulty is that at any particular spot there are particles of various sizes, but moving along the paper, certain sizes are eliminated. So in the very beginning he has all the sizes. In the next section he has gotten rid of all the big ones, so there are only the smaller ones. And in the next section there are still smaller ones, and they become smaller. I think we have an expert here that can tell more about the actual mechanics and the function of that instrument. We do not have at any one place only one particular particle size, but we have a group of particles with certain sizes eliminated from it. If I am not correct, will you correct me on that? The problem that occurs in the lung, however, is that as these aerosol particles are traveling down deeper and deeper they pick up moisture and become different. They become larger, and thus the probability of their retention changes again. So any particle that happens to come out of the cigarette is not predestined to be held at a particular area because of its particle size. Only after it has picked up its own moisture and changed to a different size will it then probably be picked up somewhere. We cannot really tell where and how much pickup there is. I will leave it open for you to ask Dr. Goetz, because he is the man that has the instrument.

MR. STEPHENSON: I think your description of the instrument was very good. Dr. Goetz is trying to simulate what happens in the lungs by having the proper humidity and particles of a particle size and then measuring the change in size. So perhaps he will be able to shed some light on this question.

DR. ALTSHULLER: What bothers me is how does any given material, be it benzo[*a*]pyrene or any other polynuclear, first of all transfer from the particle to biological tissue. We heard it stated that there are coatings of water vapor over the particles.

When this hits a site in the body, how does transfer occur? Can we ignore the fact that there may be small molecules adsorbed in the surface of the molecules—formic acid, formaldehyde, what have you? How do they effect the irritation of the site and the transfer of the large molecule from the particle to the biological site?

DR. FALK: About this problem of how they transfer from the particle to the cell, the first problem is: Will the particle stay in the lung at all? Now, you have two chances, either it will or will not. Most of the time the particles will not stay in the lung but will be expectorated and removed. The next movement is that of the phagocyte to grab it and take it away. The phagocyte has actually a very short life. It will disintegrate, and the fragments will be picked up by another phagocyte. Or they will be picked up by other cells, depending on where the phagocyte happens to disintegrate. The phagocyte does not recognize barriers at all. There are none of any sort, iron or whatever, that could occur to a phagocyte. It goes through from the circulation to the alveoli, out of the alveoli back into the circulation, or along into the rest of the tracheobronchial tree. There is simply no end to it. If the carbon particle enters a phagocyte, the only function of the phagocyte is to digest it and get rid of it. Now, the first action that occurs in this very high protein medium is that the benzo[*a*]pyrene is eluted from the soot and remains inside the phagocyte in protein solution. It stays there as long as the phagocyte lives. It may or may not be oxidized. I am not yet sure about this, whether the oxidation already occurs in the phagocyte. The soot stays in there too. Ultimately the soot is deposited either in a lymph node or elsewhere in the lung. In the autopsy room you find it there days or years later. The soot is always completely free of any polycyclic hydrocarbons. However in Russia a paper was presented in 1958 in which an investigator reported finding benzo[*a*]pyrene in the lung after hydrolysis of the autopsy material. We found practically no benzo[*a*]pyrene. If you realize that it takes no time for the protein to elute benzo[*a*]pyrene from particles of a certain size range, then it is reasonably obvious why there is not any left behind. If the benzo[*a*]pyrene is eluted and travels in its protein solution at the time it goes in the blood stream, after awhile it probably gets into the liver, gets metabolized into various conjugated phenols, and eliminated in the bile from the body. The carcinogenic process may or may not have occurred in the lung or elsewhere while the benzo[*a*]pyrene was in transit.

We are putting a great deal of emphasis on the possibility that the benzo[*a*]pyrene is retained in the body in cases where the liver is not functioning properly. I feel that liver function is exceedingly important, not only for defense mechanisms in the body but also just to get rid of that little bit of benzo[*a*]pyrene. We looked for the benzol[*a*]-pyrene in animals that had liver damage and that retained 70 percent of the injected material over 8 hours, the time that we studied it. We could not find it concentrated in any particular place. It was diffusely distributed and it was not really waiting to be excreted, because the removal of benzo[*a*]pyrene and this metabolite from the liver slowed down considerably after a certain length of time. Actually there is no bottleneck in the liver with all the benzo[*a*]pyrene piled up behind it. It finds other places to go. So, as Dr. Hoffmann pointed out, we may have to look at other properties that may contribute, such as other toxic hydrocarbons causing a change in the metabolism and therefore in susceptibility to cancer.

DR. HOFFMANN: If I understand correctly the work of Dr. Auerbach, the vast majority of the squamous-cell carcinomas of the lung has appeared in the bronchial tree, not in the alveoli. Dikun, in Leningrad, found traces of benzo[*a*]pyrene in 15 human lungs. This he determined by fluorescent spectra.

DR. LINDSEY: I believe here one should refer to the work of Sir Ernest Kennaway, who collected a number of lungs from autopsies. He destroyed all the tissue with alcoholic potassium hydroxide and then determined the carbon. Generally with a town dweller about 1 g of carbon was recoverable from both lungs. Calculation shows that if persons living in the same locality for their entire lives of 60 to 70 years were breathing atmospheres of the same composition as those breathed at the time of death, they would have taken between 100 and 200 g of carbon into their lungs.

This shows that the cilia action and other means of disposal had removed the great quantity of carbon which had gone in. Another little point, again in tribute to Sir Ernest, is that his various coworkers at the time were unable to find any benzo[*a*]pyrene in the 1 g or so of carbon which was recoverable.

DR. COMMINS: We all seem to have our own pet methods of analyzing polycyclic aromatic hydrocarbons. I am wondering if the time has come to formulate a universal method for the determination of polycyclic aromatic hydrocarbons. We all have different methods; maybe they are all correct. I cannot believe this. I am not saying that any method I have used is more correct than anybody else's, but has the time come for a universal method?

CHAIRMAN SAWICKI: Any comments? I think this is a good idea. We do need to standardize some of these methods. There is no doubt about that.

I have a question here, Dr. Commins. After you extract particulates with cyclohexane or benzene, how much organic material is left?

DR. COMMINS: Quite a lot. If you use a benzene extract following a cyclohexane extract it produces quite a lot of material, depending on the type of sample. However, we could find only traces of the hydrocarbons we were interested in. I cannot give an exact figure.

CHAIRMAN SAWICKI: Well, we have extracted with dimethylformamide at room temperature after the boiling benzene extraction for 8 hours, and our dimethylformamide turned dark brown very quickly.

DR. FALK: After benzene extraction we occasionally extracted with acetone. We obtained a goodly number of other compounds, and we identified three of them as quinones of polycyclic aromatic hydrocarbons. One was anthanthrone, one a benzo[*a*]pyrene quinone, and one a pyrene quinone. I feel they are always there, but we usually do not pick them up with benzene. Also we found an unknown phenol.

DR. COMMINS: I would be greatly interested in how you positively identified the quinones. There are several possible quinones that could form, of course, by oxidation or whatever the process. Regarding the compounds "X" and "Y," I have tried to look for compound "X." There is a compound that comes intermediate between pyrene and benzo[*a*]pyrene—we feel this might be compound "X," although the spectral data given in the paper are not adequate for a positive identification. This compound, if not separated clearly from anthracene, interferes and has a peak there which I think is at 374.5 mμ. It does not seem to be a particularly stable compound. Could you comment on this, and the other problem?

DR. FALK: We are not particularly sure of the nature of the quinones. The way we identify them is strictly by their absorption spectrum and fluorescence spectrum after reduction to the dihydroxy compound, which goes without trouble. So we have two absorption spectra, one fluorescence spectrum. If it happens to agree with any of the known literature-described compounds, I usually call that a tentative identification. I am not so sure because I have not seen all the other potential phenols and dihydroxy compounds to really be sure.

Now your question about compound "X." I was very much bothered by it because it is not stable. By the time you do even as little as running one ultraviolet absorption spectrum on a rather dilute sample, you have changed it to something else. This something else actually now fluoresces and has a spectrum of pyrene, except that all the wavelengths are wrong. You see it as showing a bathochromic shift so that it seems to be a derivative of pyrene, which is entirely unstable in ultraviolet light and goes to a compound that has a good fluorescence and absorption spectrum. I did not go beyond that in following it up, mainly because I dropped the analysis of exhaust at that time. The compound "Y" actually was only called "Y" until I knew it was the benzo[*k*[fluoranthene. I have a compound "Z," but I guess it never got into the literature.

DR. HOFFMANN: During our study on automobile exhaust-gas condensate benzene extract we obtained the column chromatography fraction that might be called comparable to this compound "X." We were able to separate it by two types of paper chromatography, mainly into benz[*a*]anthracene and 11*H*-benzo[*b*]fluorene.

CHAIRMAN SAWICKI: Thank you. We appreciate the contribution of everybody and we would like to thank you all on behalf of the U.S. Public Health Service and the Laboratory of Engineering and Physical Sciences. Mr. Stern, do you want to say anything? This is your last chance.

MR. STERN: With those words I could not possibly add anything. Thank you.

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UNITED STATES OF AMERICA

The First International Conference on The Biology of Cutaneous Cancer

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Harold L. Stewart, *M.D.*

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FOREWORD TO THE FIRST CONFERENCE ON THE BIOLOGY OF CUTANEOUS CANCER[1]

HAROLD L. STEWART, Laboratory of Pathology, National Cancer Institute,[2] Bethesda, Maryland

The First Conference on the Biology of Cutaneous Cancer grew out of an idea generated independently and nearly simultaneously in two places separated by more than 6,000 miles—Philadelphia and Tokyo. In Philadelphia in the fall of 1960, Dr. Frederick Urbach and Dr. Carroll F. Burgoon, Jr., of the Temple University Skin and Cancer Hospital, conceived the idea of a conference on skin cancer, with international participation, to bring together dermatologists and experimentalists for exchange of ideas and for planning future studies. They wrote to a number of investigators in this country and abroad and asked them to explore the possibility and submit suggestions and plans. The replies professed a real enthusiasm and contained many excellent comments. With this encouragement, the organizers arranged for a conference to be held in 1962 near the spring meeting place of the American Association for Cancer Research. In Tokyo, on October 15, 1960, on the last day of a symposium held by the Committee on Geographic Pathology of the International Union Against Cancer, R. E. J. ten Seldam, Professor of Pathology at the University of Western Australia and cochairman of the newly established Subcommittee on Geographic Pathology for the Pacific Area, suggested that cutaneous cancer be the subject of the first scientific gathering of that group. It was as clear from the data presented to participants of the symposium in Tokyo, as it was to those at the Conference in Philadelphia, that the pattern of cutaneous cancer in different parts of the world showed remarkable differences that should be thoroughly investigated. To hold such a meeting presented difficulties, not the least of which was financial. As so often happens, the areas of the world with the most challenging scientific problems possess few or no funds to support such studies or finance a symposium to plan for future investigations.

Finally, the two groups became aware of their common interest, and the present Conference was the result. These proceedings abundantly demonstrate the happy blend of disciplines and talents that this amalgamation brought together. They attest to the conviction reached in Tokyo that the pattern of cutaneous cancer varies remarkably in different regions of the world, and its proper study requires international cooperation. The present Conference has given opportunity for many stimulating and fertile discussions which can lay the groundwork for the design of joint undertakings in geographic pathology and dermatologic research. A practical solution for some of the problems discussed may hopefully follow this joint effort. Investigators from widely scattered communities have brought into perspective the variations in the prevalence and site distribution of cutaneous cancers within their world-wide distribution. These variations cannot be by chance alone but must result from causes that we can hope to disclose if scientists on the spot pursue their investigations with vigor. The Conference increased our understanding of some obscure points, but at the same time, it revealed how many large and important areas of total ignorance exist. Nevertheless, our experience with skin cancer in the past has taught us optimism. We are indebted to studies of skin cancer for much of the knowledge we now possess about cancers in general. Skin cancer was the first cancer for which a cause was demonstrated—soot, 1775. The cause of cancer of another site was not discovered for more than 100 years; it was not until 1879 that lung cancers in Schneeberg miners were shown to be caused by ionizing radiation. In the intervening years knowledge had accumulated about four other causes for skin cancer in human beings—arsenic in 1822, crude wax from coal in 1875, and coal tar and shale oil in 1876. To these causes others were added in succession; among them ultraviolet light, creosote, X rays, shale oil, mineral oil lubricants, and crude anthracene, all of which may cause skin cancer in man when applied directly to the skin.

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Of cancers with known causes that physicians see in their daily practice, skin cancer ranks first by a large margin. On the experimental side, our early knowledge about cancer research grew out of the laboratory investigations of Yamagiwa and Ichikawa who followed up the shrewd clinical observations that had been made earlier on skin cancer induced by coal tar. With this past history we have every reason to be optimistic and feel confident that the peculiar variations in histologic type and site distribution of skin cancers revealed by this Conference will yield important information on etiology. With this as a guide, the initiation of vigorous investigations by the participants should reveal new knowledge of inestimable value.

This Conference represents a landmark in the progress of dermatology. Its proceedings give us the latest information on the clinical and pathologic manifestations of cutaneous cancer in man and the research accomplishments from animal experimentation. The speakers have emphasized the practicability of studies on skin cancer and the success to be gained by focusing the attention of experienced investigators on the subject. If the geographic approach is to realize its full potentialities, it needs the support of scientists from many disciplines. Now is the time to carry out the investigations discussed at the Conference because the improvements in living conditions and other unforeseen changes all over the world will abolish our present opportunities for such enterprises. The publication of the proceedings that follow should convince everyone that we have an important and urgent duty to learn all we can about the biology of skin cancer and its geographic variations while the opportunities last.

PERCIVALL POTT'S CONTRIBUTION TO CANCER RESEARCH[1]

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WHEN PERCIVALL POTT was 62 years old he had practiced and taught surgery for 39 years. In that year of his life, 1775, he sent to his publisher a small book containing 5 surgical papers, one of which was an article (*see* pp. 7–13) on cancer of the scrotum (*1*). Historically this was the beginning of cancer research because Percivall Pott probed beyond the immediate problem of the surgical treatment of scrotal cancer into the question of etiology. He found that scrotal cancer occurred almost exclusively in chimney sweeps and ascribed its origin to contact with soot. As Pott wrote: "The disease in these people seems to derive its origin from a lodgment of soot in the rugae of the scrotum." Thus he identified the first carcinogen known to man. Not only did his discovery have practical applications for cancer prevention, but it began a chain of inquiry into the cause of cancer that a century and a half later led to the elucidation and identification of carcinogenic hydrocarbons, and the rich harvest of knowledge that has resulted from the study of chemical carcinogenesis.

The recognition of soot as a carcinogen developed from a thoughtful inquiry, and only fleeting indications of Pott's own thoughts are found in the article—for Percivall Pott, as James Earle wrote: ". . . studiously avoided reference to obscure and general principles—and preferred reasoning by analogy and induction from established facts . . ."(*2*). It is not often appreciated that cancer was accurately diagnosed 187 years ago, particularly when the science of cellular pathology had not been developed, but the clinical description of the progression of scrotal cancer in chimney sweeps, written by Pott, leaves no doubt concerning the accuracy of the diagnosis. Further, scrotal cancer in chimney sweeps was not the usual form of cancer and Pott pointed this out quite clearly: ". . . all this makes it (at first) a very different case from a cancer which appears in an elderly man whose fluids are become acrimonious from time, as well as other causes, or from the same kind of complaint in women who have ceased to menstruate."

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Pott was impressed with other clinical aspects of chimney sweeps' scrotal cancer and wrote: "I never saw it under the age of puberty,[3] which is I suppose one reason why it is generally taken both by patient and surgeon for venereal, and being treated with mercurials, is thereby soon much exasperated." Mercurial therapy, the chief treatment for venereal diseases, was applied locally as an ointment. Pott probably observed these unresponsive patients with scrotal lesions were all chimney sweeps by trade. He noted that the chimney sweeps themselves referred to the scrotal lesion as a "soot wart," and it is reasonable to assume that his attention was directed more to the idea of a disease associated with a specific occupation—a concept not unknown to him, for he refers in scholarly manner to Ramazini's book "de morbis artificum," the Colic of Poictou, and the disorders of white lead workers. This scholarly method was important, for it provided a new approach: Scrotal cancers were in some way connected with the occupation of the patient.

Pott was a scholar though he had only 9 years of formal education before his apprenticeship to the surgeon Edward Nourse (*2*). Sir James Earle wrote of his father-in-law: "He often said he began to teach when he had much to learn; and as he was not actuated by that opinionative wisdom which sometimes attends advanced life, after all his study and experience he confessed that he still retained a long list of inquirenda." Pott's epitaph reads: "The labours of the ancients were familiar to him; he scorned to teach a science of which he had not traced the growth" (*2*). On the same subject, Sir James Earle said, "Mr. Pott always professed great value and respect for the early writers on the art and perused their voluminous works with great diligence and sagacity."

Strengthened by evidence and by the precedent of other occupational diseases, Pott must have then continued his inquiry beyond the environs of his usual routine. While older members of the lucrative chimney sweeps' trade had contact with soot in many different ways, such as sifting and preparing soot for sale, the most intimate contact was that of the small boy who climbed the tortuous, narrow English chimneys. Since Percivall Pott was aware of the abuse of these children (he vividly described their "singularly hard fate"), it can be assumed that *some* time about 187 years ago he observed chimney sweeps at work. He must have been struck by seeing soot in the rugae of their scrotums, and then in that 18th century moment have perceived for the first time a process which we now speak of as "chemical carcinogenesis."

The recognition of soot as a carcinogen was a monumental step in our knowledge of carcinogenesis and Pott's paper presented just this idea to his contemporaries, for they immediately began to see the principle involved in other circumstances. Benjamin Bell in 1794 (*3*) wrote: "This variety of cancer appears obviously to be produced by soot, for it is found that besides chimney sweeps those who are employed in manufactures in which soot enters as an ingredient are occasionally seized with it." In 1808 James Earle recorded the history of Allan Spragg, a 49-year-old gardener who developed an epithelioma on the dorsum of his left hand. This gardener had spread soot in his garden one spring 5 years before to kill slugs. He carried the soot in a pot that was hung over his left hand; the handle rubbed spilled soot onto his wrist. Earle grasped the point that skin cancer elsewhere than in the scrotum might be caused by soot. Henry Earle, a grandson of Percivall Pott, found "some exceptions to the rules" of his grandfather (*4*). He agreed that the "peculiar stimulus" soot caused scrotal cancer, but it was not the only factor; he said that the frequency in chimney sweeps was not great, considering how many were exposed. Earle suggested that a ". . . constitutional predisposition is required which renders the individual susceptible to the action of the soot." He strengthened the idea with facts; scrotal cancer sometimes favored "different branches of the same family at a particular period of their lives." Heredity in cancer was not a new subject, for John Hunter discussed this question in 1787 in his Lectures on the Principles of Surgery (*5*).

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[3] James Earle later showed Pott an 8-year-old apprentice chimney sweep with a scrotal cancer (*2*).

Further, Henry Earle had difficulty in understanding why individuals as children were exposed to soot and then developed cancer after the age of 30. By 1823, soot cancer in chimney sweeps was provoking new questions that today would be debated in terms of genetic factors predisposing to cancer, the latent period of carcinogenesis, etc.

Chimney sweeps' cancer was considered by Astley Cooper, T. B. Curling (*6*), and James Paget (*7*). Curling took up Henry Earle's concept of the individual predisposition and hereditary background. He described a sailor with a scrotal cancer in the fifth decade of life. The sailor had been "brought up as a sweep" but had been to sea for the 19 years preceding the appearance of the neoplasm and during this time had no contact with soot. In 1856 James Paget raised many new questions about scrotal cancer. He described precancerous changes, but, concerning soot, said, "I suspect that the true influence of the soot in this disease is not that its continued contact determines the growth of cancers but (at least in part) that it produces a state of skin which provides an apt locality for epithelial cancer in persons of cancerous diathesis."

In 1892 Henry Butlin reinvestigated in detail the subject of scrotal cancer in chimney sweeps (*8*). Though a century had passed since Pott's death, new facts had come to light and old ones, such as the continuing appearance of scrotal cancer in chimney sweeps still remained.

The expanding Industrial Revolution had brought an increased need for lubricating oils for machines. Prior to 1850, machines were lubricated with animal oils. With the rise of the mineral oil industry and after the reports of Volkmann in 1875 (*9*) and Bell in 1876 (*10*) on the occurrence of scrotal cancer in the tar, paraffin, and shale oil workers, Butlin recognized other crude carcinogens for the scrotum. He also recognized a few other unpleasant facts: "We who live in large cities swallow and inhale soot every day in greater or less quantity. We accept the position, grumblingly no doubt, still we accept it; we know that our great smoke fogs make many people ill, and that they kill a certain number with acute disease. But it is possible that we owe far more than this to the influence of *floating soot* and that a part of the increase in the occurrence of that awful disease, cancer, of which the national statistics tell so striking a tale, is due to the daily contact of soot with areas of the lining of the mucous membrane, or to the entrance of soot into some one or other of the internal organs, in which the conditions are favorable to its action." This complaint about increasing deaths from cancer was dated July 9, 1892!

Butlin brought out an important concept from his review. There were several types of carcinogens for man: soot, tar, paraffin, and certain mineral oils, and he raised an important question: If these agents could cause cancer on the skin, might they not also cause cancer internally? In this century great additions to our knowledge of carcinogens for man have been published by Henry (*11*) and by a participant in this Conference, Wilhelm Hueper (*12*).

Pott's contribution is being extended in another way and the realizations of this work may be the most important of all. For tar, soot, and crude mineral oils induce cancers not only in man but also in many other species. The historic achievement in 1915 of Yamagiwa and Ichikawa is well known [*see* Bibliography in (*13*)]. Passey in 1922 extracted from soot a potent carcinogen for mice (*14*). The brilliant series of experiments of Kennaway, Heiger, Mayneord, Cook, and Hewitt from 1922 to 1935 led finally to the identification of many active principles of several crude products. Sir Ernest Kennaway described the historic development of these important experiments in 1955 (*15*).

All these experimental techniques and agents have led to a new order of investigation which relates to the all-important question of how these chemicals transform normal cells to neoplastic ones. But most of all, experimental evidence has given irrefutable validity to the speculation of Butlin that the agents which cause cancer on the skin can also induce cancer internally. The mechanism of action of soot or its active ingredient is not understood, even after 187 years of enormous, technological development, and the easiest, most effective method to control scrotal soot cancer is the same as that available to Percivall Pott and his contemporaries: *prevention by avoidance of contact.* It is remarkable that Percivall Pott did not suggest this method.

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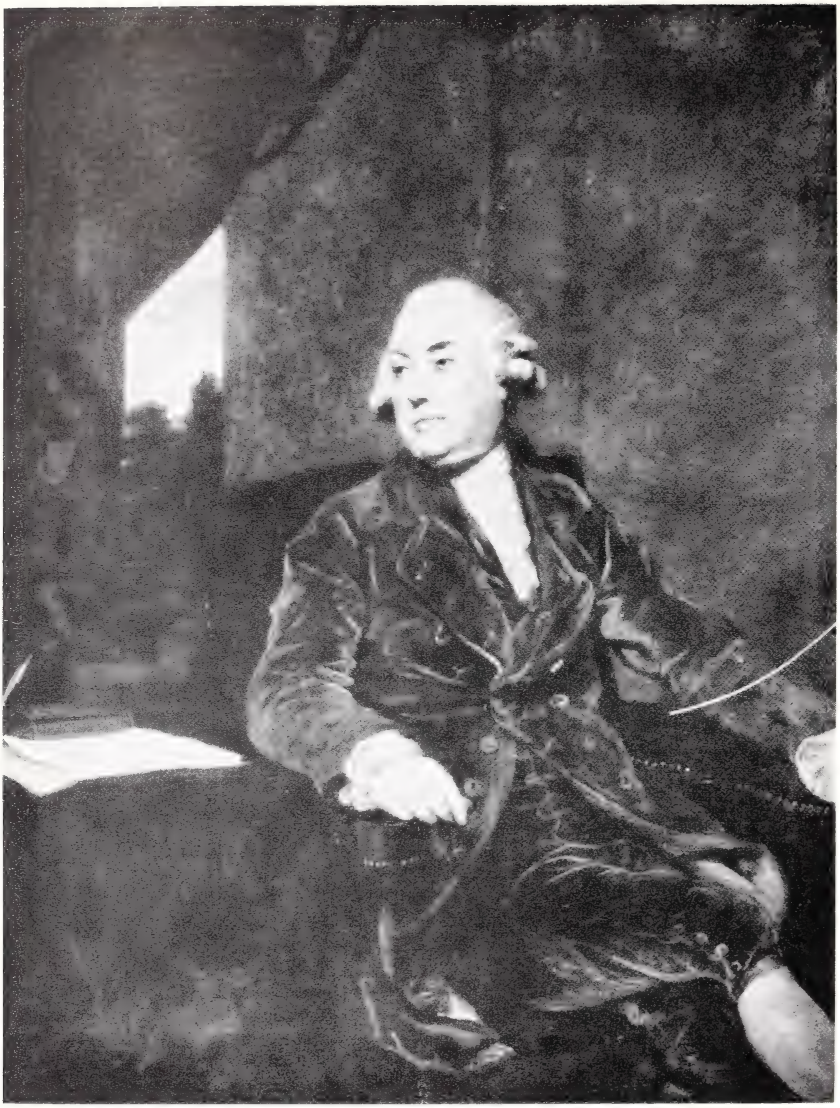

Percivall Pott (1714–1788)

Portrait by Sir Joshua Reynolds (1784)

This painting now hangs in St. Bartholomew's Hospital, London, where Percivall Pott was a surgeon, and is reproduced here by permission of the Governors of the Hospital.

CHIRURGICAL OBSERVATIONS

Relative to

The Cataract,
The Polypus of the Nose,
The Cancer of the Scrotum,
The Different Kinds of Ruptures,
and
The Mortification of the Toes and Feet,

By PERCIVALL POTT, F.R.S.
Surgeon to St. Bartholomew's-Hospital.

LONDON,
Printed, by T. J. Carnegy,
For L. Hawes, W. Clarke, and R. Collins,
in Pater-noſter Row. M dcc. lxxv.

CANCER SCROTI.

RAMAZINI has written a book de morbis artificum; the Colic of Poictou is a well-known diſtemper, and every body is acquainted with the diſorders to which painters, plummers, glaziers, and the workers in white lead, are liable; but there is a diſ-

eaſe

eaſe as peculiar to a certain ſet of people which has not, at leaſt to my knowledge, been publickly noticed; I mean the chimney-ſweepers' cancer.

It is a diſeaſe which always makes its firſt attack on, and its firſt appearance in the inferior part of the ſcrotum; where it produces a ſuperficial, painful, ragged, ill-looking ſore, with hard and riſing edges. The trade call it the ſoot-wart. I never ſaw it under the age of puberty, which is, I ſuppoſe, one reaſon, why it is generally taken, both by patient and ſurgeon, for venereal, and being treated with mercurials, is thereby ſoon, and much exaſperated: in no great length of time, it pervades the ſkin, dartos, and membranes of the ſcrotum, and ſeizes the teſticle, which it inlarges, hardens, and renders truly and thoroughly diſtempered; from whence it makes its way up the ſpermatic proceſs into the abdomen, moſt frequently indurating, and ſpoiling the inguinal glands: when arrived within the abdomen, it affects

fects ſome of the viſcera, and then very ſoon becomes painfully deſtructive.

The fate of theſe people ſeems ſingularly hard; in their early infancy, they are moſt frequently treated with great brutality, and almoſt ſtarved with cold and hunger; they are thruſt up narrow, and ſometimes hot chimnies, where they are bruiſed, burned, and almoſt ſuffocated; and when they get to puberty, become peculiarly liable to a moſt noiſome, painful, and fatal diſeaſe.

Of this laſt circumſtance there is not the leaſt doubt, though perhaps it may not have been ſufficiently attended to, to make it generally known. Other people have cancers of the ſame parts; and ſo have others, beſide lead-workers, the Poictou colic, and the conſequent paralyſis; but it is nevertheleſs a diſeaſe to which they are peculiarly liable; and ſo are chimney-ſweepers to the cancer of the ſcrotum and teſticles.

If there be any chance of putting a ſtop to, or preventing this miſchief, it muſt be by the immediate removal of the part affected; I mean that part of the ſcrotum where the ſore is, for if it be ſuffered to remain until the virus has ſeized the teſticle, it is generally too late even for caſtration. I have many times made the experiment; but though the ſores, after ſuch operation, have, in ſome inſtances, healed kindly, and the patients have gone from the hoſpital ſeemingly well, yet, in the ſpace of a few months, it has generally happened, that they have returned either with the ſame diſeaſe in the other teſticle, or in the glands of the groin, or with ſuch wan complexions, ſuch pale, leaden, countenances, ſuch a total loſs of ſtrength, and ſuch frequent and acute internal pains, as have ſufficiently proved a diſeaſed ſtate of ſome of the viſcera, and which have ſoon been followed by a painful death.

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If extirpation ever bids fair for the cure of a cancer, it ſeems to be in this caſe; but then the operation ſhould be immediate, and before the habit is tainted. The diſeaſe, in theſe people, ſeems to derive its origin from a lodgment of ſoot in the rugæ of the ſcrotum, and at firſt not to be a diſeaſe of the habit. In other caſes of a cancerous nature, in which the habit is too frequently concerned, we have not often ſo fair a proſpect of ſucceſs by the removal of the diſtempered part; and are obliged to be content with means, which I wiſh I could ſay were truly palliative; but here the ſubjects are young, in general in good health, at leaſt at firſt; the diſeaſe brought on them by their occupation, and in all probability local; which laſt circumſtance may, I think, be fairly preſumed from its always ſeizing the ſame part: all this makes it (at firſt) a very different caſe from a cancer which appears in an elderly man, whoſe fluids are become acrimonious from time, as well as other cauſes; or from the ſame kind of complaint in women who have ceaſed to menſtruate. But be all this as it may, the ſcrotum is no vital organ, nor can the loſs of a part of it ever be attended with any, the ſmalleſt degree of inconvenience; and if a life can be preſerved by the removal of all that portion that is diſtempered, it will be a very good and eaſy compoſition; for when the diſeaſe has got head, it is rapid in its progreſs, painful in all its attacks, and moſt certainly deſtructive in its event.

THE BIOLOGY OF CUTANEOUS CANCER: INTRODUCTION AND HISTORICAL SURVEY[1]

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INTRODUCTION

Having heard Dr. Potter's admirable account of Percivall Pott and Skin Cancer, there is little more for me to say from the historical point of view for our minds are now turned irresistibly to the future.

As all dermatologists know from experience, the skin is the defense system of the body with major protected observation posts (sense organs) and sentinels (nerve terminals) widely distributed.

As all investigators of cancer know, of all cancers cutaneous cancers are the most numerous and the most easily detected and studied directly. The skin provides more obvious precancerous lesions than any other system of the body. If these lesions are treated many cancers can be prevented. The skin can give indications of functional states and disorders of deep-lying organs and these symptons must never be ignored.

Little wonder, therefore, that a highly productive marriage is consummated between skin and cancer, and resulted in this great Skin and Cancer Hospital of Philadelphia and in the Barnard Free Skin and Cancer Hospital where I used to be.

Little wonder, also, that Alan Gregg, the distinguished Director of Medical Sciences of the Rockefeller Foundation, devoted so much energy and so many dollars in an effort to establish Departments of Dermatology and of Psychiatry in medical schools when he returned to New York after long service in Paris. In an article in the *Journal of the American Medical Association,* Gregg placed dermatology high on the list of medical fields ripe for development. Yet there has been a strange lethargy in the Public Health Service when the promotion of dermatology is urged. The idea seems to be that diseases of the skin are seldom death-dealing, except for smallpox, which can be absolutely prevented, so why worry.

Dr. Rothman's book, entitled *The Physiology and Biochemistry of the Skin,* has established the basis for the new dermatology.

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

As the great Philadelphian dermatologist, Dr. F. D. Weidman, has said, "If there is any part of the body which literally thrusts the evidence of its advancing years upon whomsoever may look, it is the skin." A child knows the significance of wrinkles, gray hairs, and baldness. Employers, prospective husbands and wives consider the skin the indicator of youth. Untold millions are spent in attempts to keep the skin youthful; this is not done for any other system of the body. Yet the enormous cosmetic industry has not risen to the occasion by generously supporting research on the skin.

However, inspection of the skin comes first in all physical examinations the world over. Allergy vies with carcinogenesis. The two-stage concept of carcinogenesis has been worked out primarily with epidermis. How much of this concept applies to mesenchymal carcinogenesis is important to determine, as we shall learn from Dr. J. W. Orr, our revered British colleague. Plastic surgery did wonders during the war, owing to great advances in skin grafting—now we have skin banks.

In preparing material for the first edition of my book on *Special Cytology,* I placed the chapter concerning the skin first, where it belongs, and asked Dr. Charles Stockard of Cornell University College of Medicine to write it. This is the same Stockard who added a young Greek doctor named Papanicolaou to his staff in Anatomy. At this time Stockard was studying vaginal smears from guinea pigs. He presumably inspired Papanicolaou. But Stockard could not find time to write this chapter, so I took over, and the cutaneous defense system has held my mind captive ever since.

There are many intriguing things about the skin and skin cancer, which are certainly well known to this audience. On this occasion, however, I may be pardoned if I mention some that seem to me outstanding in connection with the efficiency of this defense system and the onset of malignancies.

The skin is, of course, divisible into an outer, thin avascular epidermis and an inner, thicker mesenchymatous dermis. Time permits only brief reference to the epidermis, because it is in this layer that three major types of cancer develop from three kinds of cells.

Indeed this epidermis is almost unbelievably thin in protected areas like the antecubital space, where in vertical sections from young males it is only about 34 μ thick. If we allow for shrinkage, it would be definitely less than 100 μ—about the diameter of a human egg—the limit of particle size visible to the naked eye.

Within this short distance there is an effective transition from multiplying epidermal cells within to dead, tightly bound cells that make up the surface. "While we are in life we are in death," is a true saying.

Within this epidermal layer, which is thicker in some places, is a deprivation gradient from within outward, for the cells are progressively deprived of life-giving oxygen and nutriment until they die.

The chief point I would emphasize is that these thin layers of frontier cells are subject to vital hazards, greater and more diversified than any other cells of the body. Burning heat, shriveling cold, radiant energy, mechanical injury, invading microorganisms and viruses are but a few of them. The stage is set for innumerable mutations, some malignant—perhaps even for the birth of viruses. The epidermis has been styled the happy hunting ground of viruses, so many different ones are found in it.

This epidermal investment is integrative as well as protective because the entry of some substances is permitted, and information about the environment is transmitted to deep-lying tissues and organs by the sentinels and sense organs. Breaches in it are promptly repaired. It is the privilege of dermatologists to keep it healthy and vigorous.

Lastly the epidermis—with the dermis on which it lies and the epidermal appendages, hairs, sebaceous and sweat glands—reflects by its behavior many vitally important conditions in the body as a whole. One of these is fascinating and mysterious. I refer to acanthosis nigricans (patches of gray-black warty masses, especially in the axilla), repeatedly observed in some patients who have visceral cancer, particularly stomach cancer.

Gregg was right in believing that dermatology is an alluring field of activity. The complexity of the skin, together with its regional differences coupled with easily distinguished areas of rapid and slow aging, challenges an analysis of its activities and ills. It is the system *par excellence* most exposed to external environment and reflecting activities within, so that cutaneous cancer is the most fruitful field for studies on the geographic pathology of cancer. People of all nations consider cancer our number one, and most implacable, enemy. The only reason why the World Health Organization has been so tardy in the investigation and prevention of cancer is that even now no way has been found to prevent it wholesale, like malaria and malnutrition.

They say that new techniques are keys to scientific treasure, and which rust away and are of no value whatsoever unless used. We have many key techniques which will open the way for scientific investigation of the skin: The separation of epidermis cleanly from dermis in a condition suitable for chemical analysis has proved fruitful. Dr. D. R. Coman and his associates here in Philadelphia have employed the Chambers technique of micromanipulation to measure the force necessary to separate pairs of normal and malignant epidermal cells. This could be used to measure the effectiveness of influences designed to promote cohesion of the cells and thus perhaps keep carcinomas *in situ* from spreading. Electron microscopy, with wonderful clarity, reveals the intimate connections between cells and might provide structural controls for such experiments. The chemical interpretation of minute structural details is advancing by the enzymatic removal of ribonucleic acid and other components susceptible to enzymatic digestion. What we need most to discover is the nature of the contents of areas that have very little electron density and look empty.

Methods of tissue culture of epidermal cells are being utilized as never before. Epochal discoveries have been made in the discovery of nucleic acid viruses. In following them up, tissue cultures, clonal in nature, will be of the greatest assistance. The riddle of the source of melanocytes has been solved. Coons' technique for the histochemical identification of antigens has proved effective. Many new ways of approaching fundamental problems are available.

In the mesenchymatous dermis the chemical advances have been encouraging and the role of the dermis in the production of cutaneous cancers, long insisted on by Orr, is coming into its own. Recent investigations of cell movements in tissue culture reveal that in invasion malignant cells almost feel their way. The phenomenon of contact inhibition can be said to have been discovered. I could continue but time passes and old people are inclined to garrulity.

We, assembled here, see good omens for the future. First and foremost this is stated to be the first conference on the biology of cutaneous cancer—an implication that there will be others. We hope that in these others the participants from abroad will be as well chosen, for I cannot imagine a more effective international group than is now assembled.

This program begins with the aging background of cutaneous cancers of which I shall have much, perhaps too much, to say later.

Nearly half of the program is devoted to what we commonly call the geographic pathology of skin cancer. There are many intriguing attempts to discover why there are such remarkable differences in various geographic areas. Now that the comparisons are being made with great accuracy, with consideration of all known variables, it is anticipated that outstanding discoveries concerning the etiology of cancer will be made; for here nature is the experimenter and exposures that would be highly dangerous for us to employ experimentally are made. It is nice to see such a fine group of investigators assembled from around the world, some of whom are old friends of mine.

About half of the program is devoted to experimental cutaneous carcinogenesis in animals which is, on the contrary, purposeful and unrestricted. In this field I also find many investigators whose names are known around the world. Many of them I am proud to say are friends of mine. Cutaneous cancers are, as I have said, pre-eminent in the cancer problem. Dr. Potter has clearly described the investigations of Percivall Pott, and we remember that our Japanese friends were the first ones to produce cancer in mammals. It is the hope and expectation that this first conference on the biology of cutaneous cancer will clarify the susceptibility of human beings everywhere to carcinogens, now tested only on animals because it is unthinkable that we test them directly on man.

Last, but by no means less effective and enjoyable, is the fact that this program gives us such wonderful opportunities to talk over these problems when we get ideas—at meals, at the symphony, at the theatre, and during visits to points of historical interest in the great city of Philadelphia.

You all know that this Conference has been organized and is being conducted by the pioneering Skin and Cancer Hospital of Philadelphia. We expect that others, like us, will be inspired by this approach to illuminate some of the problems of skin and cancer for which this great hospital was chartered and in which the investigations of Dr. C. F. Burgoon, on viruses, Dr. F. Urbach, on tissue oxygen tension, as well as clinical studies by them and others, are eminent. That Merck Sharp & Dohme should generously sponsor this international project is also auspicious. It is timely and shows vision of a field of human welfare that is now fortunately attracting world-wide attention.

DIFFERENTIATION AND REDIFFERENTIATION IN SKIN [1,2]

CHARLES BREEDIS, Department of Pathology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

SUMMARY

Embryonic ectoderm and mesoderm unite to form the primitive skin, becoming epidermis and dermis, respectively. Cells from the neural crest migrate in to form the pigmentary system. In different regions the early epidermis develops into specialized structures, such as hair follicles, glands, and epidermal ridges, apparently in response to still earlier specializations of the dermis underlying these regions. This interaction between epidermis and dermis continues throughout life, so that in general the perfect regeneration of a region of lost skin epithelium depends upon the integrity of the underlying dermal structure. However, it can be shown that in wounds, under certain conditions, both epidermis and dermis retain a degree of their former embryonic plasticity, so that all cells of the wound epithelium may be equipotential in producing such structures as hair follicles, while regions of the granulation tissue of the wound may become specialized to produce dermal papillae.—NCI Monogr. No. 10: 21–31 1963.

SOURCES OF EPIDERMIS, DERMIS, AND PIGMENT CELLS

EARLY IN embryonic life prospective skin ectoderm and mesoderm come in contact, presumably because there is some specific affinity between the cells of the two kinds of tissues. The tissues are at first in direct contact, but later a visible cementing material, the basement membrane, comes between them. All of the embryonic ectoderm has the potentiality of forming skin epithelium, but due to various migrations and the action of local inductors some of it forms such structures as lens, conjunctiva, parts of teeth, etc.

The dermis is derived partly from the mesoderm of the somatopleure and partly from the dermatotome of the mesodermal somites. Precursors of pigment cells move into the skin from the neural crest. This movement also seems to depend upon some kind of tissue affinity. The movement continues until a final equilibrium in distribution of pigment cells is reached (*1*).

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

[2] This work was made possible by grant C-1116 from the National Cancer Institute, National Institutes of Health, Public Health Service.

REGIONAL DIFFERENCES AND THEIR ORIGIN

In different regions the ectoderm undergoes modifications to form epidermal appendages such as scales, feathers, hair, nails, and glands. The distribution and form of these structures are genetically determined. It would be of interest to know whether these modifications arise because of some specialization within the ectoderm itself or are determined by some specialization in the underlying mesoderm. In amphibia many ectodermal organs such as gills and teeth are induced by reactions originating in the mesoderm. These inducing influences may be highly specific in organizing the ectoderm or they may merely activate it nonspecifically.

In feather formation the first visible change is the formation of a dermal papilla in the mesoderm. Epidermal proliferation follows. However, if the ectoderm over a prospective dermal papilla is removed, the papilla does not form (*2*), which suggests that both kinds of tissue must be present for the initiation of differentiation of either. Also, there seem to be quantitative as well as qualitative factors involved in this interaction. If mesoderm that normally produces feathers is transplanted under a region of ectoderm that normally does not, feather germs form in the region (*3*). Similarly mesoderm from a presumptive foot bud grafted beneath wing ectoderm causes the formation of a claw on the wing instead of a wing feather. However, as the epidermal tissue becomes organized it seems to become dominant over the dermis, and if a sufficient amount of it is then transplanted to a new site it retains its old characteristics (*4*).

Induction in the embryos of mammals has not been as thoroughly studied as it has been in birds and amphibia, but it appears likely that similar mechanisms are operating. Hardy (*5*) observed tissue cultures of embryonic mouse skin and found that, if epidermis and dermis were cultured unseparated, normal hair follicle development resulted. In the absence of mesoderm no epidermal thickenings occurred and in the absence of epiderm there was no formation of dermal papillae.

REGENERATION OF SKIN TISSUES IN ADULT MAMMALS

It has been known for a long time that when adult human skin is injured the epithelium will not regenerate perfectly unless the specialized structures of the dermis have been preserved. Bishop (*6*) found that scar tissue was formed when a layer of skin experimentally removed included the papillary layer and the bases of the hair follicles, whereas epidermis regenerated normally if these tissues remained. Similarly, in wounds and burns involving the volar surfaces of the palms and fingers, the epidermal ridge pattern re-forms unless the injury is deep enough to destroy the dermal ridge pattern. For every specialization of the epidermis there is apparently a complementary specialization of the dermis, and usually the latter must remain uninjured if perfect regeneration of destroyed epidermis is to occur.

However, regeneration need not be perfect, and from this point of view one may ask: To what extent can new formation of destroyed structures occur? The question has been controversial for years. Our experiments in this direction were the outcome of incidental observations. An attempt was made to obtain a purely squamous sheet of scar epithelium in the rabbit for the purpose of inoculating it with the Shope papilloma virus. Circular wounds were made on the backs of rabbits and were hindered or prevented from contracting by means of metal splints (*7*). The total skin thickness, including the panniculus carnosus, was removed. Few of the resulting scars were free of hair follicles, and, in some, follicles were present in great profusion. The distribution of these new follicles differed from that in either the embryo or adult rabbit. In the 3-week embryo the follicles are readily identifiable (fig. 1). If a stained preparation of epidermis, separated by Cowdry's method, is made at this stage, the follicles are distributed throughout the epidermis in a geometric pattern (fig. 2) which persists. New follicles arise from wound epithelium quite differently. Here the epithelium develops bars of ridges of thickening, usually radially directed, and the new follicles arise from these, first near the periphery of the scar and later at the center. Figure 3 shows the follicles arising as buds from the bars, figure 4, the mature follicles.

Since some contracture of the wounds often occurred despite the metal splints it was desirable to prevent contracture entirely. This was done by means of a splint, which is illustrated in figure 5. It consists of a ring cut out of sheet tantalum, with a notch cut out of its inner edge. A disc of skin is removed down to the panniculus carnosus, its diameter such that when the ring is inserted between the panniculus carnosus and the overlying dermis the wound edge lies on the ring, and then the wound edge makes contact with panniculus only at the notch. The wound epithelium, derived entirely from the region of the notch, eventually covers the entire exposed panniculus carnosus. Figure 6 shows a section through a wound healed in this way, photographed through crossed polaroid filters. The dense, doubly refracting collagen bundles of the old skin at the edge of the wound appear bright. The granulation tissue within the wound area has none of these bundles. Numerous hair follicles developing from the wound epithelium are also shown in higher power in figure 7.

The bars of thickened wound epithelium from which the new follicles arise (fig. 3) might have been derived largely from the epithelium of cut hair follicles at the edge of the wound. Argyris (*8*) has found that follicle epithelium contributes to wound epithelium in the mouse and we have seen this occur in the rabbit. Such epithelium might be especially capable of redifferentiating into follicles, but if so, then it must also have been capable of inducing the formation of new dermal papillae below it. Hence it seemed likely that hair follicles in the adult rabbit would be capable of redifferentiating into complete follicles if their bulbs and dermal papillae were cut away.

It was possible to give a fairly convincing demonstration that this was so. The melanin pigment of black rabbits is confined to active hair follicles. If a hair growth cycle is induced by plucking, the newly activated follicles grow deep into the dermis and become easily visible. One can then turn back a flap of skin and cut away the bases of the follicles, including the dermal papillae, under a dissecting microscope. This operation is illustrated in figure 8. After the operation the hairs in this region fell out and the region became depigmented, while the surrounding region continued in the active growth phase (fig. 9). The epithelium of the undercut region became extremely hyperplastic (fig. 10) and later numerous new hair follicles developed from it (fig. 11). The redifferentiation began at the periphery of the undercut region and proceeded toward the center (fig. 12). Eventually the population of new follicles was as dense or more dense than that in the original skin.

Billingham and Russell (*9*) have observed neogenesis of hair follicles in wound epithelium of rabbits in which wound contracture failed to occur. They noted in black rabbits that the new follicles were not pigmented. Kligman and Strauss (*10*) later reported regeneration of vellus hair follicles in human skin that had been abraded for cosmetic purposes. Straile (*11*) attempted to repeat our experiments of 1954, but was unable to prevent extensive wound contracture and did not observe neogenesis of follicles. Boulas (*12*) observed neogenesis essentially under Billingham's conditions but in addition was able to dissociate granulation and epithelization. Topically applied hydrocortisone inhibited the granulation tissue to such an extent that the wound epithelium migrated practically over the panniculus carnosus. Later, when epithelization was complete, much granulation tissue formed between the muscle and the epithelium. Of great interest is the finding of Billingham *et al.* (*13*) that the skin of the deer antler, which re-forms each year, develops new hair follicles. Joseph and Townsend (*14*) found that wounds made on the inner surface of the ear of the rabbit did not contract. The tissues that filled such a defect became organized into essentially new skin, complete with hair follicles and rete pegs.

The sebaceous gland apparently develops whenever a hair follicle has developed. When the gland in the mouse is destroyed by the application of methylcholanthrene, a new gland develops from the external root sheath (*15*). After injury, the human sweat gland can regenerate to some extent. The cells of the secretory coil have the ability to make new secretory cells, basal and luminal cells of the sweat duct, a spiraling sweat duct unit, and mature epidermal prickle cells (*16*).

The foregoing observations are consistent with the thesis that all the dividing cells of adult mammalian skin epithelium are equipotential. The various, apparently different, basal cells of the hair follicles, glands, and surface epidermis would then not be true differentiations but rather modulations in the sense of Weiss (*17*). The dermis, too, must contain equipotential elements adaptable to changes in the overlying epithelium. These potentialities of skin are probably not of much practical importance in the healing of large, accidentally incurred wounds. Contracture and scar formation almost invariably occur, but for understanding the nature of skin, and possibly its neoplasms, they may turn out to be extremely important.

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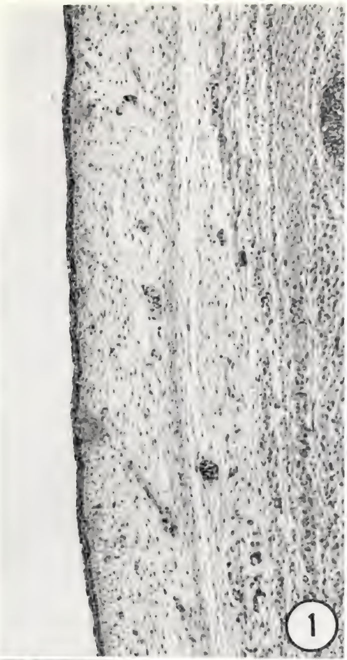
Plate 1

Figure 1.—Section through skin of back of 21-day rabbit embryo. The thickened regions of epidermis on *left* are beginning hair follicles. Under each a dermal papilla is beginning. Hematoxylin and eosin. × 70

Figure 2.—View from above of epidermis from back of a 21-day rabbit embryo. Epidermis has been removed by acetic acid method of Cowdry and stained with hematoxylin. Darkly staining early hair follicles correspond to epidermal thickenings seen in figure 1. × 70

Figure 3.—View from above of epithelium from healed wound, 31 days after removal of full thickness of skin. Bars of thickened epithelium running *vertically* in illustration show more densly stained regions which represent beginning hair follicles. Hematoxylin. × 34

Figure 4.—View from above of epithelium from healed wound, 34 days after removal of full thickness of skin. Many mature hair follicles are seen arising from bars of thickened epithelium. Hematoxylin. × 34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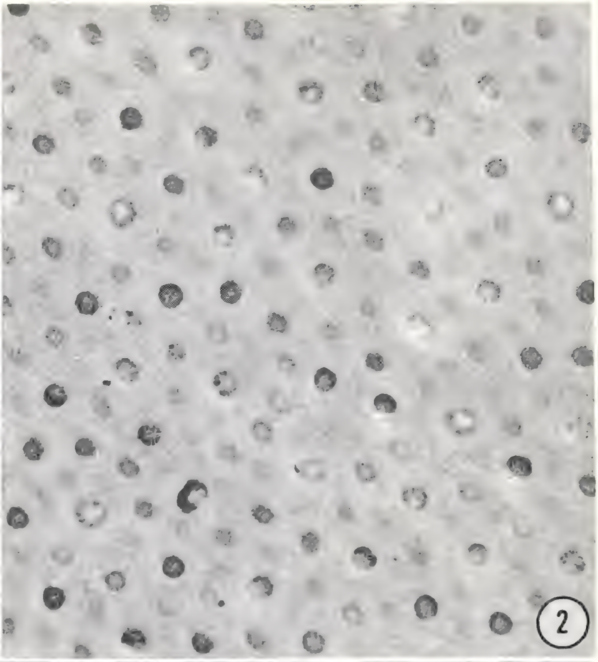
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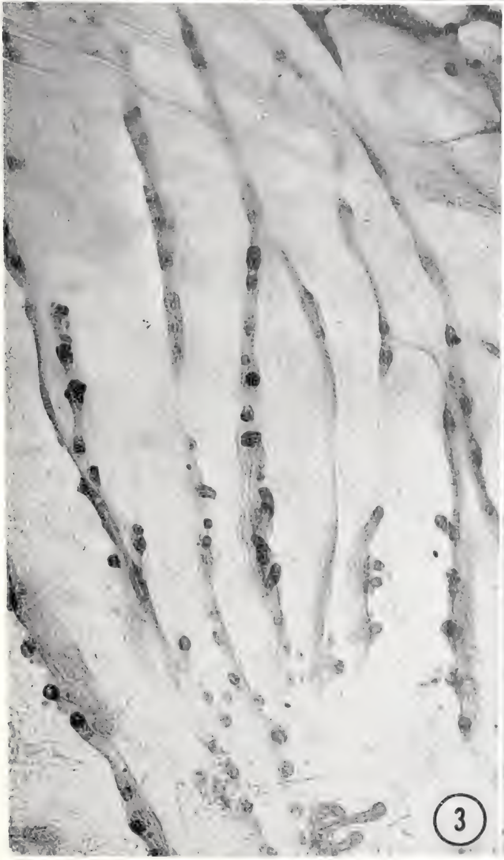
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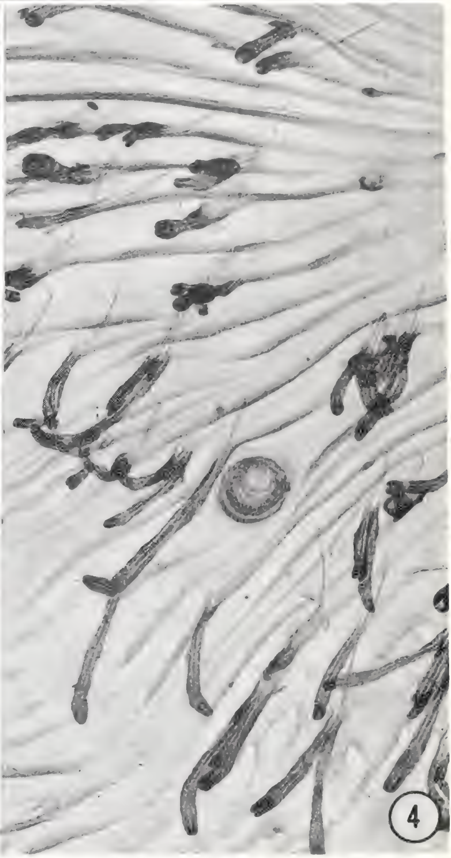
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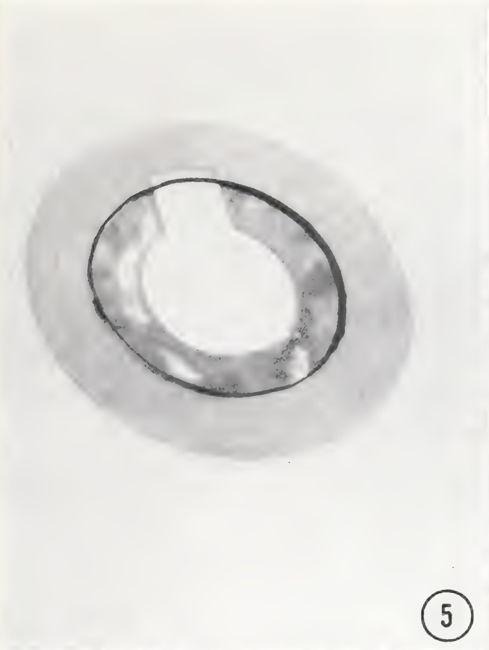
Plate 2

Figure 5.—Tantalum ring used to prevent contraction of wounds during epithelization, shown in simulated position in the skin. The circular ring is cut from 0.007 inch thick surgical sheet tantalum The outer diameter is 42 mm, the inner 16 mm. A notch, 8 × 8 mm, was cut from inner edge. In the rabbit, the ring lies between the panniculus carnosus and the dense collagen of the dermis. In the photograph a layer of translucent plastic simulates this overlying skin. The hole in the plastic represents the wound. The edge of the wound lies on the tantalum except in the notch where it contacts the panniculus carnosus. All of the wound epithelium arises from this region of contact and migrates until it covers the exposed panniculus. × 1.2

Figure 6.—Section through a wound healed for 43 days under conditions shown in figure 5. Photograph was taken through crossed polaroid filters, one behind the specimen and the other in front. If the page is turned sideways the skin surface is on top. At *left,* the strongly anisotropic collagen bundles of old skin at edge of wound appear bright. Some of this collagen also lies on panniculus carnosus below the wound but none is seen in new granulation tissue that has filled the wound. Many hair follicles are regenerating from the wound epithelium. Small defect in wound epithelium is an artifact. Hematoxylin and eosin. × 7.5

Figure 7.—Higher magnification of figure 6. × 70

Figure 8.—Method of removing bases of growing hair follicles and their dermal papillae. A hair growth cycle was induced by plucking 11 days previously. A flap of skin was reflected and panniculus carnosus is shown from below. A circular defect was cut in panniculus carnosus and the bases of many hair follicles were cut away with surgical eye instruments. × 1



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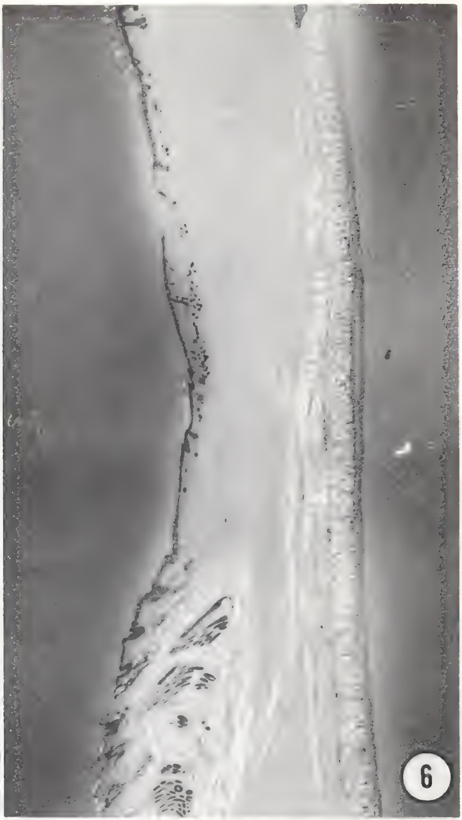

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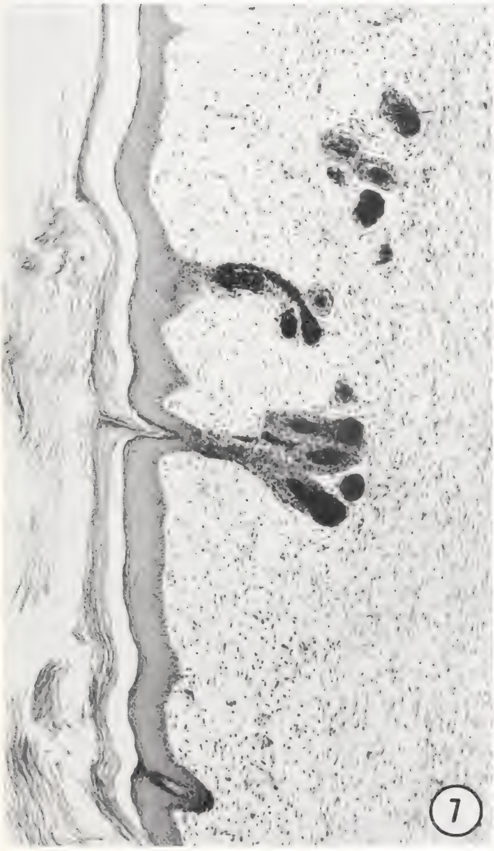

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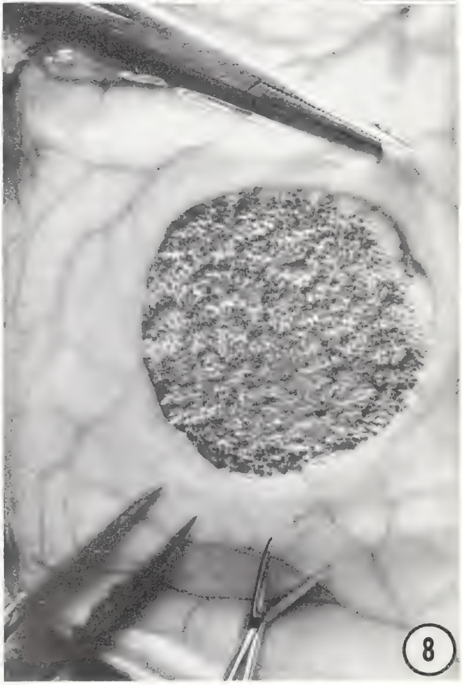

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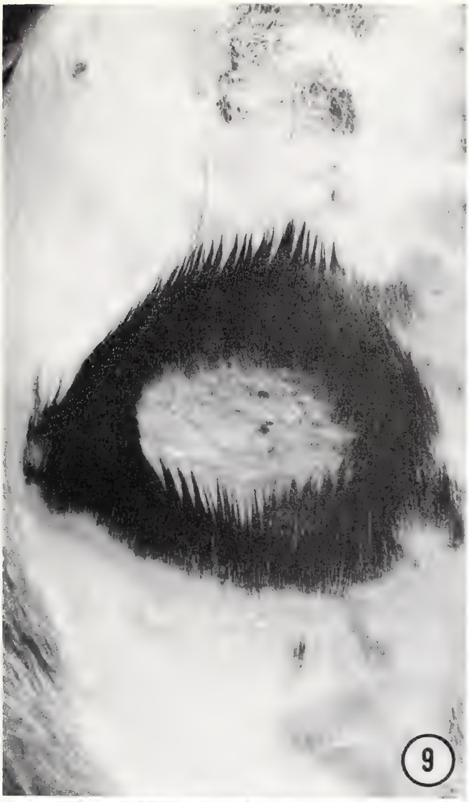
PLATE 3

FIGURE 9.—View of surface of skin 10 days after the undercutting operation illustrated in figure 8. The pale, hairless region surrounded by hair-bearing skin shows the area in which the operation had been performed. Hairs of the undercut follicles have fallen out and follicles have lost their pigment. × 1

FIGURE 10.—Section through center of region of skin from which bases of hair follicles were cut away, 15 days after the operation. Epithelium is extremely hyperplastic. Hematoxylin and eosin. × 40

FIGURE 11.—Section between center and edge of region of skin from which bases of hair follicles were cut away, 15 days after the operation. Epithelium is hyperplastic and many new follicles are seen arising from bases of cut follicles. Hematoxylin and eosin. × 40

FIGURE 12.—View of surface of skin 24 days after the undercutting operation shown in figure 8. Black hairs are growing in area of skin where operation was performed. They are most pronounced at periphery of this region and diminish toward the center. × 1



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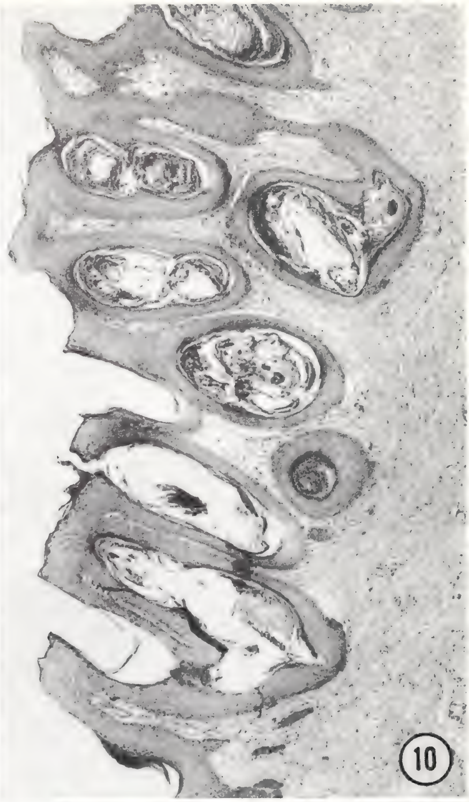

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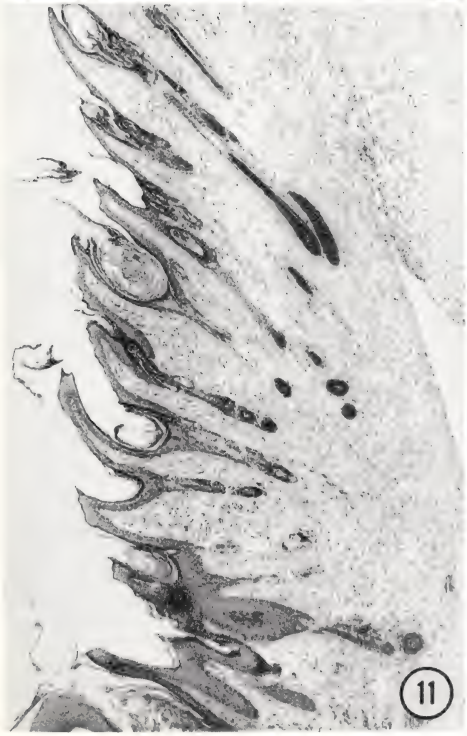

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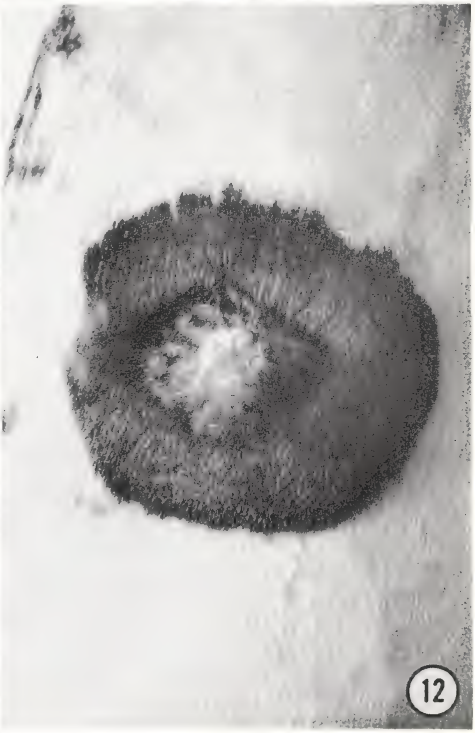

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HAIR GROWTH CYCLES AND SKIN NEOPLASIA[1]

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SUMMARY

Hair growth cycles can profoundly affect tumorigenesis. In the rabbit, multiple applications of 7,12-dimethylbenz[*a*]anthracene (DMBA) result in benign hair-follicle tumors if they appear in the resting phase of the hair cycle. But papillomas arise if the tumors appear in the growth phase. Incidence of virus-induced tumors is also affected by the hair cycles. Application of the Shope papilloma virus to skin in the growth phase results in many more tumors than if the papilloma virus is applied in the resting phase, even though the damage produced by scarification during the application of the virus is the same in both phases. In mice a single application of DMBA to the dorsum results in many skin tumors if the skin is in the resting phase at the time the carcinogen is applied. Similar treatment to skin in the growth phase results in few tumors. Whether this enhanced tumor production in resting skin is due to the more severe damage produced in resting skin and/or to the reported prolonged retention of the carcinogen by resting skin is not clear. Finally, recent evidence suggests that the increased tumor production in resting skin may be due to changes occurring in the "promotion" phase of skin tumorigenesis and not to changes in the "initiation" phase.—NCI Monogr. No. 10: 33-43, 1963.

THIS PAPER relates the hair growth cycles of mammals to the evolution of skin neoplasia. The paper will be divided into two parts. The first will briefly discuss the normal pattern of hair cycles, and the second will indicate how the hair growth cycles influence the evolution of skin neoplasia.

HAIR GROWTH CYCLES

It is assumed that all mammals have hair growth cycles. In those studied, the pattern of the cycles has been shown to vary considerably.

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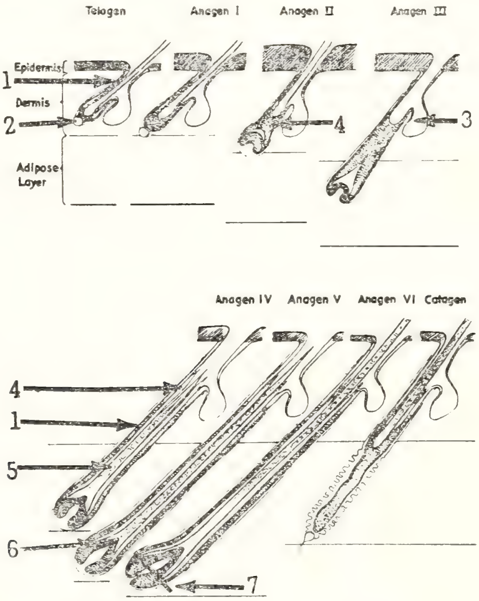
In some, the hair growth cycles sweep over the body in waves, whereas in others each hair follicle has its own growth cycle. In still others, there may be gradations of these two extremes (*1*, *2*). It is best first to review the hair growth cycle in the mouse, since it has been the most extensively studied, and then to compare the cycles of other mammals to it.

The hair growth cycle of the mouse is divided into three stages: growth (anagen), resting (telogen), and a transitional stage between that of growth and rest (catagen) (*1*, *2*). These stages are illustrated in text-figure 1, *see also* bold numbers in text.

In resting skin, the epidermis is 1 to 2 cell layers thick. The resting hair follicle is composed of a club hair surrounded by a single sheath, the external root sheath: **1**. The base of the sheath just below the club hair is the germ region, and beneath this is the dermal papilla: **2**. Each hair follicle has an associated sebaceous gland: **3**. The resting hair follicle is embedded in the dermis, below which is the subcutis or fatty layer.

Approximately 24 hours after initiation of hair growth (anagen I), either spontaneously or by plucking, there is a burst of mitotic activity in the germ region. Within 2 to 3 days (anagen II) the mitotically active germ region envelops the dermal papilla, and, as illustrated in text-figure 1, the internal sheath is formed: **4**. Concomitant with these changes in the hair follicle, the epidermis enlarges due to cell hypertrophy and hyperplasia. Three to 4 days after the initiation of hair growth (anagen III), the hair follicle grows into the subcutis, keratinization



TEXT-FIGURE 1.—Stages of the hair growth cycle in the mouse (*2*). For explanation *see* text. *Bold numbers* in text relate to those shown in this text-figure.

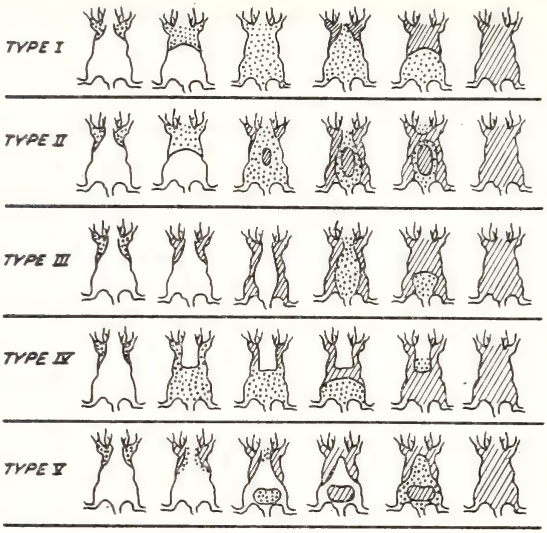
begins, and pigment appears in the bulb area. In addition, the dermis and subcutis thicken. By 6 days (anagen IV) pigmentation, **6**, of the hair shaft, **5**, is well under way. Proliferation of cells for keratinization is largely restricted to the matrix: **7**. The epidermis decreases in thickness, and in contrast, both the dermis and subcutis continue to increase in size (anagen V). For the next 8 to 10 days (anagen VI) the growing follicle shows no further morphogenetic changes and is largely concerned with the production of the hair shaft, which it produces at the rate of approximately 1 mm per day. During this time the epidermis is thinner than it is in the resting phase: **1**. The dermis and subcutis, however, attain their maximum width. At 18 to 20 days (catagen) hair production and pigmentation cease, and the lower half of the follicle degenerates. The dermal papilla moves upward and comes to lie underneath the new germ region. The epidermis, dermis, and subcutis return to the size they were in the resting phase.

In pigmented mice, such as the C57BL, one can monitor the underlying histological events by noting the changes in skin color. Resting skin is pink for 1 or 2 days after inititation of hair growth when it becomes white. After about 4 to 5 days the skin becomes light blue due to pigment synthesis in the hair follicles that is visible through the partially transparent skin. During the next few days it becomes darker blue and finally black. By 8 to 9 days the new hair shafts are visible through the surface of the skin. On completion of hair growth, the skin changes from black to light gray at the beginning of the transitional phase. Upon its reentrance into the resting phase the skin is again pink. In albino mice one cannot tell the presence of the growth phase until the new hairs begin to protrude through the surface of the skin.

The pattern of hair waves in pigmented mice has been studied extensively by Chase (*3*). The initial wave of hair growth (G_1), although not strictly a wave, begins in the dorsum, goes to the ventrum, and finally to the extremities. In contrast, the second wave, G_2, begins in the ventrum and moves dorsally and posteriorly. This basic pattern is repeated for 2 more hair generations, G_3 and G_4. In G_5 the first obvious breakdown of the hair waves is seen. Hair growth often becomes limited to islands of growth. With increasing age the islands become more numerous and the general pattern of hair growth more varied.

In albino mice, the succession of hair waves has been studied in some detail by Borum (*4*) whose findings are similar to those of Chase and Eaton (*3*). In addition, Borum presents a more detailed account of the variations that can be seen in the pattern of the hair cycles as early as G_2. Text-figure 2 shows that 5 patterns of hair growth can exist in G_2. Type I represents the usual wave movement across the back. Yet this occurs in only 58.3 percent of the females and 85.0 percent of the males. Thus, patterns of hair growth are not uniform, even in young mice and, additionally, sex differences occur.

The hair growth cycles of the rat appear to be basically similar to those of the mouse (*5, 6*). The waves are said to sweep from the ventrum



GRAPH I
Various types of hair patterns in G_2.
The drawings represent the dorsal aspect of the mice. The dotted areas indicate sites covered with short, white, not yet outgrown hairs of G_2; their presence was ascertained by ruffling the black-dyed coat. The shaded areas indicate sites in which G_2 had grown out; in dyed mice these sites were silvery grey. The six drawings in each row illustrate six developmental stages of G_2 as a function of time.

K. Borum
Acta Pathol. Microbiol. Scand.
XXXIV : 521-541,1954.

TEXT-FIGURE 2.—Various hair patterns in G_2 of the mouse hair growth cycle. Drawings are of the mouse dorsum. *Dots* indicate areas where new hairs are protruding through the skin surface. *Shaded areas* indicate fully grown hair follicles. *Unmarked areas* indicate resting hair follicles.

dorsally, progress anteriorly and then posteriorly. Supposedly, waves occur every 34 days (*5*). Plucking initiates hair growth in the rat, as in the mouse, and can be used to bring an area of skin into a single phase of the hair growth cycle. Beyond these facts little else is known about the hair cycles in the rat.

The hair cycles in the rabbit occur annually or semiannually (*7*). The waves sweep from the dorsum to the flanks (figs. 1 through 8). The ventrum and limbs appear to have their own hair cycles. As in the rat and mouse, hair cycles may be initiated by plucking. The histological changes after initiation of hair growth appear to be similar to those in the mouse (*8*). There is an initial burst of mitotic activity in the germ region followed by elongation of the follicle, envelopment of the dermal papilla, and formation of the internal sheath. The epidermis enlarges, but there is no definite information as to whether the dermis and subcutis thicken. Once the new hair follicles are differentiated, the follicles primarily produce hair at the rate of 1 mm a day. This begins about 10 to 12 days after initiation of hair growth. Also at this time the epidermis returns to the thickness it has in the resting phase.

Except for mice, rats, and rabbits, few mammals have had their hair cycles investigated. A particularly pressing problem is an analysis of the hamster hair cycle since the hamster is being used considerably for experimental skin research.

In contrast to the previously mentioned mammals, the hair cycles in the guinea pig and man do not occur in waves but are said to be limited to a single hair follicle (*2*, *6*). There seem to be regional differences such as in the head, where 90 percent of the hair follicles are usually in the growth phase, although both resting and growing follicles can be found in any given area. In the leg about 45 percent of the follicles are in the growth phase. Although they undergo changes similar to those seen in rodents, there does not seem to be any change in the thickness of the epidermis, dermis, or subcutis. However, this may be due to the fact that this problem has been insufficiently studied.

The principal lessons the skin cancer investigator can learn from the knowledge of hair cycles are that they obviously drastically affect the structure of the hair follicles and that in mammals such as the mouse all the components of the skin are affected. Moreover, in the mouse, in which we are beginning to get detailed information about the patterns of the hair cycles, we find that the patterns are complex, variable, and unreliable. It should be possible for one to control hair cycles initiated by plucking. Since Chase (*1*) has clearly demonstrated that the events after plucking are the same as those after the spontaneous initiation of hair growth, plucking apparently is a satisfactory technique for controlling the hair cycle and thereby the conditions within the skin.

EFFECT OF HAIR GROWTH CYCLES ON SKIN TUMORIGENESIS

Before discussing the relationship of hair cycles to skin neoplasia, let us see if the presence of hair follicles affects skin tumorigenesis. In hairless mice, skin tumors have been reported to appear spontaneously and to be increased after application of a carcinogen (*9*, *10*). Presumably, then, skin tumors can appear in the absence of hair follicles. On the other hand, Lacassagne and Latarjet (*11*) have stated that in scars produced by ultraviolet irradiation, which are free of hair follicles, tumors do not appear after treatment with methylcholanthrene. However, in the edges of the healed wounds where hair follicles are present, tumors appear. Therefore, the experiments of Lacassagne and Latarjet (*11*) would argue that hair follicles are necessary for skin neoplasia to occur. Although the results of Deringer (*9*), Montagna and Chase (*10*), and Lacassagne and Latarjet (*11*) appear to be opposed, this may not be the case. In the experiments of Lacassagne and Latarjet (*11*) not only are the scarred areas free of hair follicles, but the dermis has been changed. Thus the lack of tumors may be due to changes in the dermis. That the dermis may play a role in the control of the function of skin epithelium and in skin carcinogenesis has been suggested by a number of investigators (*12*, *13*). On the other hand, hairless mice are not completely free of hair follicle epithelium (*10*). What is actually lacking is the organization of the epithelial cells into follicles. The presence of hair follicle remnants may be sufficient to result in tumorigenesis. These studies more properly suggest that organized hair follicles are not needed for tumorigenesis to occur in the skin. Thus the question of whether hair follicles are necessary for skin tumorigenesis must be kept open. Indeed we may find that hair follicles are necessary for some kinds of tumors and not for others. We may now turn to the question of whether hair cycles can affect skin tumorigenesis.

Multiple applications of 7,12-dimethylbenz[*a*]anthracene (DMBA) result in the production of tumors in rabbit skin irrespective of whether the skin is in the resting or growing phase (*14*). However, the kind of tumor varies with the stage of the hair cycle in which the tumor is detected. If the skin is in the resting phase, a "self-healing" tumor develops and regresses when the hair follicles enter the growth phase. A benign epidermal papilloma occurs if the tumor develops during the growth phase. Occasionally both tumors may become invasive. Virus-induced tumors of rabbit skin also are affected by hair cycles (*8*). If the Shope papilloma virus is applied during the growth phase, many more tumors arise than if the virus is applied in the resting phase. The amount of damage produced by scarification when the virus is applied is the same in both stages.

Obviously the hair cycles can affect the course of tumorigenesis in mammals. The question arises as to how the hair cycles affect skin tumorigenesis. To get insight into this problem, we must turn to the work with mice.

As early as 1951 Wolbach (*15*) suggested that skin tumorigenesis in mice is affected by the stage of the hair cycle at the time of application of carcinogen. Andreasen and Engelbreth-Holm (*16*) and Borum (*17*) have studied this problem in detail in an inbred strain of albino mice (ST/Eh).

Text-figure 3 shows clearly that after a single application of carcinogen, skin of mice in the resting phase develops many more tumors than that in the growth phase. Borum (*18*) has also investigated whether the hair cycles affect tumor growth after the tumors appear. A technique was developed by which a cast was made of the tumor. By filling in the casts of the tumor with cement, removing and weighing it, Borum obtained a relative measure of increase in mass and changes in size. The author found that when a wave of hair growth sweeps over an area of skin bearing a tumor, the growth of the tumor is enhanced. In addition, in many instances the shape of the tumor is also altered. These studies clearly suggest that in mice the hair cycles can affect the number of tumors that appear after treatment with carcinogen and also their future course.

The question arises how the stage of the hair cycle at the time of initial application of carcinogen affects the incidence of tumors. Histological studies by many investigators have revealed that a single application of carcinogen such as 20-methylcholanthrene (MCA) or DMBA causes considerable changes in resting, but not in growing skin (*19–22*). A single application of a carcinogen on resting skin within 5 days results in a

	Females		Males	
	A. & E.-H.	Borum	A. & E.-H.	Borum
Number of mice with papillomas/ number of mice in the growth phase	5/26 = 19.2 %	0/23 = 0 %	1/30 = 3.3 %	0/32 = 0 %
Number of mice with papillomas/ number of mice in the resting phase	22/34 = 64.7 %	25/26 = 96.3 %	15/34 = 44.1 %	15/19 = 81.0 %

K. Borum
Acta Pathol. Microbiol. Scand.
XXXIV : 542-553,1954.

TEXT-FIGURE 3.—Number of papillomas appearing on the backs of mice treated with a single application of DMBA in the growth or resting phase of the hair growth cycle. The figure compares the data of Andreasen and Engelbreth-Holm (*16*) with those of Borum (*17*).

marked epidermal cell hypertrophy and hyperplasia, loss of hair and sebaceous glands, and the conversion of the hair follicles into hyperplastic cords of enlarged cells. Similar treatment of skin with growing hair follicles (specifically in anagen V or VI) results only in loss of sebaceous glands and mild epidermal enlargement. The reason for the greater sensitivity of resting skin to the carcinogen is not known. Preliminary work by Borum (*23*) using C^{14}-labeled DMBA suggests that the amount of carcinogen absorbed initially is the same for growing and resting skin. Berenblum *et al.* (*24*) claim that resting skin retains the carcinogen for a longer time than growing skin, which could explain the increased sensitivity of resting skin to carcinogens. However, even if there are differences in the initial absorption and/or retention of the carcinogen, they may not be important in the eventual production of skin tumors.

To determine if the initial effects of the carcinogen are important, Berenblum *et al.* (*24*) applied DMBA in paraffin oil to skin of mice. This procedure supposedly initiates tumorigenesis but requires the action of a promoting agent, *e.g.*, croton oil, for tumors to appear. If it is the initial application of the carcinogen that is important, then resting skin treated with an initiating dose of DMBA followed by promotion with croton oil should show more tumors than growing skin treated in the same way. The results clearly show that the number of tumors that appear are the same. Thus it seems that the initial differential response of growing and resting skin may not be critical in the explanation of the greater incidence of tumors in resting skin.

In summary, we can say with confidence that hair cycles have been shown to affect the initiation and evolution of skin neoplasia in mice and rabbits. Beyond this there is little else known. When we say skin with growing hair follicles shows fewer tumors than skin with resting hair follicles after a single application of carcinogen, we really should say that skin with growing but already differentiated hair follicles (growing hair follicles in anagen VI) shows fewer tumors. We have no knowledge as to how growing skin in the early stages of the growth phase of the hair cycle will respond when the hair follicles are undergoing differentiation (anagen I–IV). That differentiating hair follicles may respond differently to trauma than growing but already differentiating hair follicles has been clearly demonstrated (*25*). We still have no clear notion of the mechanism for the differential response to a carcinogen of skin in various phases of the hair growth cycle. We do not know how far hair cycles will affect skin tumorigenesis produced by multiple applications of carcinogen or if the evolution of all kinds of skin tumors is equally affected by the hair growth cycles (*26*, *27*). It would be interesting to ascertain if the incidence of X-irradiation-induced skin tumors (*28*) is affected by the hair growth skin cycles, especially since it has been demonstrated that growing skin is more sensitive to X-irradiation-induced trauma than resting skin (*1*, *25*). Finally, and of most importance, we would like to know if the incidence of spontaneous tumors is affected by the hair cycles, a problem open to experimental attack since we can induce and control hair growth cycles by plucking (*1*, *2*).

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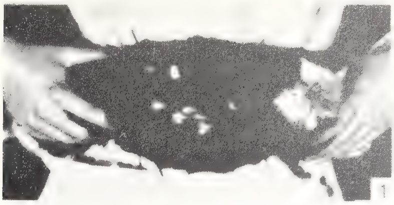
Plate 4

Pattern of hair growth in the albino rabbit. The resting skin is dyed black. Therefore *white areas* indicate new hair growth.

Figure 1.—Resting skin on dorsum of rabbit.

Figures 2 through 8.—Progression of the wave of hair growth indicated by appearance of undyed hair.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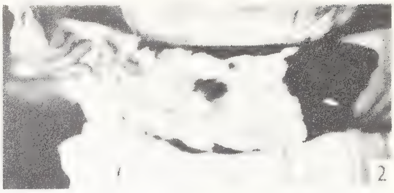
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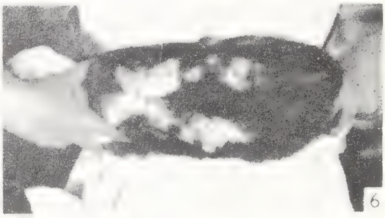
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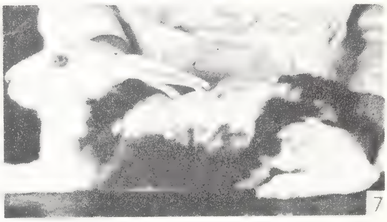
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H.J. Whiteley and F.N. Ghadially
J. Anat. 88: 13-18, 1954.

BIOCHEMISTRY AND PHYSIOLOGY OF THE SKIN [1,2,3]

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SUMMARY

Of the wide variety of cutaneous metabolic processes, certain ones are either unique for the skin or have special significance for its metabolic or physiologic function. A survey of arbitrarily selected aspects of keratinization, skin surface lipide formation, and of connective tissue, mucopolysaccharide and carbohydrate metabolism is given.—NCI Monogr. No. 10: 45–54, 1963.

OF THE awesome array of cutaneous metabolic processes, those which are either unique in the skin or have special significance in relation to it include keratinization; skin surface lipide formation; cutaneous connective tissue, mucopolysaccharide and carbohydrate metabolism; sweat production; and melanin pigment formation. In this necessarily brief survey I can only touch upon a few arbitrarily selected aspects of the first three of these subjects.

Under the influence of so far very poorly understood factors, undifferentiated epidermal cells may differentiate in one of three general directions to partake in either keratin formation, sebum production, or sweat production. Of these three directions of differentiation, keratinization seems to be the most fundamental one, and under conditions of exposure to a wide variety of injurious stimuli epidermal cells show a particular propensity to keratinize.

KERATINIZATION (*4*)

In this process essentially globular proteins of epithelial cells are converted into tough, rather chemically inert elastic fibrous proteins called keratins that are the chief constituents not only of the surface cornified

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[3] [*See* references (*1–3*).]

layers of the skin, but also of hair and nails. In considering keratins, one has to keep in mind that this term refers to crystalline fibrous proteins which can be obtained by various purification procedures from keratinous structures and that these structures contain many other extractable substances that need not be chemically bound to keratin molecules. Furthermore, keratins are a very large group of compounds with varying composition and structure. Even from a single hair, at least 4 keratins of differing chemical composition and toughness can be isolated. Keratins are distinguished by their general low solubility and great resistance to hydrolysis by either proteolytic enzymes or by dilute acids and alkalies.

In composition, two distinctive features of keratins are their high cystine sulfur content and their lack of hydroxyproline. In incompletely keratinized structures considerable sulfur in the form of cysteine and methionine may also occur. In general, the harder and more enzyme-resistant keratins have higher contents of sulfur in disulfide form than do softer keratins. On the basis of detailed X-ray diffraction studies, Astbury and his colleagues in the early 1930's discovered that keratins exist in two mechanical stereoisomeric forms, which he called α and β (*5*). Stretching the α-form by more than 50 percent yields the β-type of keratin structure. Release of stretching tension leads to reconstitution of the α-form. At first it was suggested that the polypeptide chains of keratin are folded in the α-form. More recently coiling in helix fashion, as suggested by Pauling and Corey (*6*), is a more favored view of the shape of α-keratin polypeptide chains. Because of similar X-ray diffraction behavior, Astbury classified keratins among fibrous proteins together with myosin, fibrin, and epidermin as the so-called KMEF group of proteins.

Keratins achieve their great mechanical and chemical stability through a very extensive system of primary and secondary cross-links between neighboring polypeptide chains and their branches. The primary and most significant cross-links are disulfide bonds. Hydrogen bonds between the polar side groups especially of serine, threonine, glutamic acid, aspartic acid, and arginine are also important especially for the dry strength and α-coiling of keratins. Serine and arginine are characteristically abundant in keratins. Saltlike linkages and less well-defined Van der Waal's forces are still other factors involved in the stabilization of the molecular structure of keratins.

In the process of keratinization, not only is there transformation of cytoplasmic proteins into keratin fibers but also a disintegration and elimination of cell nuclei and many cytoplasmic components. Profound dehydration also accompanies the process so that keratinized structures rarely contain more than 10 percent water.

The formation of fibrous protein in human epidermis begins in the cytoplasm of cells in the basal layer in the form of wisps of fine filaments having α-keratin X-ray diffraction properties. In higher cell layers these fibrous elements increase to form prominent tonofibrils. These fibrils have little disulfide content and are not sufficiently resistant to proteolytic enzymes to qualify as genuine keratin. Unna almost 60 years ago suggested that tonofibrils may serve as crystallization centers of keratin formation (*7*). More recently it has been suggested that sulfur-containing amino acids or peptides become incorporated into these sulfur-poor fibrous elements in the keratogenous zone of the epidermis to yield true keratin. In fact, rapid incorporation of radiosulfur-labeled cystine in the keratogenous zones of growing hair has been observed within minutes after intravenous injection (*8*). The extent to which hydrolytic decomposition of these fibrillar as well as other epidermal proteins may occur prior to the incorporation of disulfide linkages is a much debated, yet still unresolved question (*9*). The formation of disulfide cross-linkages in the keratinization process is believed to be specifically catalyzed by copper on the basis of Marston's observation of defective wool formation in copper deficient sheep (*10*).

In summary, the currently popular view is that keratins are duplex structures arising from the embedding of a primary system of filaments in a matrix of short chain polypeptides rich in cysteine residues whose conversion into cystine stabilizes the formation.

In the cellular decomposition and reorganization accompanying keratinization, lipides become freed from lipoproteins, cholesterol undergoes esterification (*11*, *12*), and phospholipides become almost completely decomposed (*13*). Also various water-soluble components of cytoplasmic and nuclear origin become degraded and/or liberated and new water-soluble compounds arise. This complex array of nonkeratin components of keratinized structures includes, for example, free amino acids, glycogen, pentoses, purines, α-pyrrolidone carboxylic acid, and intermediates of the Krebs urea cycle (*14–16*). Poorly defined epidermal mucopolysaccharides also are found in keratinized structures (*17*). It has become increasingly evident that many of these nonkeratin constituents do not merely represent biochemical debris but may have important synergistic actions with keratins in carrying out the essentially protective function of the cutaneous horny layer. Many very active enzyme functions have also been demonstrated in the keratinizing parts of the epidermis. Alkaline phosphatase (*18*), deoxyribonuclease (*19*), and arginase (*20*), in particular, are conspicuous in areas of active keratinization.

The role of the prominent particulate elements, keratohyalin and trichohyalin in the keratinization process is still not quite clear. Although by electron microscopy these granules appear to represent generation sites for keratin fiber formation, they are not always a necessary prerequisite for keratin formation. In ichthyosis in man as well as on the rat's tail where a rather hard type of epidermal keratin is encountered its formation occurs without the presence of such granules. Curiously, treatment with vitamin A that generally has so-called antikeratinizing properties causes the appearance of keratohyalin granules in these situations as well as the formation of a softer keratin (*21*).

A concept of superkeratinization has been proposed by Rothman (*22*) to describe the formation of harder epidermal keratins with diminished capacity for physiologic desquamation. Such superkeratinization is observed in ichthyosis, calluses, and precancerous actinic keratoses. Exposure of the skin to mechanical, chemical, or actinic irritation favors superkeratinization. Among chemical agents that promote keratinization, so-called acnegenic materials such as multichlorinated naphthalenes (halowaxes) and other halogenated aromatic compounds deserve attention. A most remarkably potent agent of this type is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a condensation product of trichlorophenol (*23*). A single application of 0.3 μg of this material to a rabbit's ear causes excessive follicular and surface epithelial keratinization (*24*).

SKIN SURFACE LIPIDES (*25, 26*)

Skin surface lipides originate from two sources, sebaceous-gland cells and keratinizing-epidermal cells. Morphologically, sebaceous glands are generally appendages of the hair follicles and open to the skin surface via the pilosebaceous canal. They usually have a multiple acinar structure and produce sebum by a holocrine mechanism. Mitotic activity in undifferentiated epithelial areas of these glands gives rise to a continuous stream of sebaceous cells that move from peripheral to central parts of the gland. As the cells move in this stream, they grow progressively larger as the result of intracytoplasmic accumulation of numerous droplets of fat which remain separated by a protoplasmic meshwork in contrast to the single large fat droplet seen in body depot fat cells. Finally, in the centers of gland acini the cells become so distended with fat that their cell walls disintegrate liberating the enclosed lipide. Other cell constituents which have undergone disintegration in the process are also released. Histologic observations indicate that most of the lipide that accumulates in sebaceous cells does not arise by reorganization of cytoplasmic constituents of the cells, but rather by accumulation and modification of precursor materials taken in from the outside. Endocrine factors are key agents in regulating the size and activity of sebaceous glands. Androgenic steroids in conjunction with a pituitary factor (*27*) provide the prime stimulus for sebum production.

Chemically there are vast differences between skin surface lipides and body depot fat. The latter consists mainly of triglycerides of palmitic, stearic, and oleic acids with only small amounts of other lipides, whereas the surface fats in man contain about 30 percent of unsaponifiable material, and the fatty acids are present to the extent of about 60 to 65 percent. These fatty acids occur in much richer variety and are esterified not only with glycerol but also with sterols and wax alcohols. Also in contrast to depot fat, which has essentially the same composition in all mammalian species, many striking species differences exist in the surface lipides of various mammals (*5*). During the past 15 years intensive work, especially in man, in elucidating the chemical constituents of skin surface lipides has been done. These studies opened new chapters in lipide biochemistry, abolished the concept that only fatty acids with even numbers of carbon atoms occur in natural lipides, and stimulated work that uncovered the intermediary metabolism of cholesterol synthesis. The names of Rothman, Weitkamp, Nicolaides, and their co-workers and of MacKenna, Wheatley, and associates have been especially prominent in this analytical work.

Fatty acids on the skin surface occur in the free state as well as esterified with glycerol, wax alcohols, and sterols. The relative percentage of free fatty acids is variable. This variability is no doubt caused by progressive lipolysis that occurs on the skin surface largely as a result of lipolytic enzyme activity. Microorganisms on the skin surface possess such lipolytic activity (*28*) and there is good histochemical evidence for the presence of lipases in sebaceous-gland ducts (*29*).

The first detailed study of the fatty acids in human-hair fat was carried out by means of amplified distillation (*30*). A homologous series of normal straight-chain acids was found with members from C-7 to C-22 present. Fatty acids with even numbers of carbon atoms occurred in much larger amounts than those with odd such numbers. Unsaturated acids were also found that differ from those present in commonly occurring fats. More recently, by means of gas-liquid chromatography, these findings have been confirmed and small amounts of single and multiple-branched acids throughout the series have also been detected (*31*).

Squalene is the principal hydrocarbon present in human surface lipides and accounts for between 5 and 10 percent of its composition. It has been shown to be a key intermediate in the biosynthesis of cholesterol. Nicolaides and Rothman found that the squalene content of hair fat in the adult is 3 to 4 times higher than in children, whereas the reverse is true for cholesterol content (*32*). Furthermore, it has been shown by radioisotope tracer experiments (*33*) that the main site of cholesterol synthesis in the skin is the keratinizing epidermis, while the main site of squalene synthesis is the sebaceous gland. It appears as though cholesterol synthesis by sebaceous-gland cells in man is blocked at the intermediate squalene stage. Furthermore, cholesterol synthesis in the epidermis largely follows a pathway differing from the predominant one which occurs in the liver and many other organs in that it utilizes esterified intermediates and involves formation of 7-dehydrocholesterol rather than desmosterol near the end of the biosynthetic pathway (*26*, *34*). The former compound is the precursor of vitamin D into which it is converted by appropriate ultraviolet irradiation. Large amounts of 7-dehydrocholesterol are present in lipides of living human epidermal cells (*35*). Attempts to assign special roles to squalene other than that of sterol precursor in the metabolism of human skin have so far been unsuccessful (*36*). Although there is some evidence that horse smegma has carcinogenic properties (*37*), irradiated human skin surface lipides have not been found to be carcinogenic (*38*).

The wax alcohols of human skin surface lipides are chiefly straight-chain compounds forming a homologous series ranging from C-14 to C-26 which show variations similar to those seen in the fatty acid series. All wax alcohols are found in esterified form on the skin surface. Evidence has been brought forth that the biogenesis of the various fatty acids and wax alcohols in surface fats involves the normal process of acetate addition, but that this process may commence upon the carbon skeletons of decarboxylated and deaminated amino acids so that products with odd numbers of carbon atoms and branched end groupings can result (*39*).

CUTANEOUS CONNECTIVE TISSUE, MUCOPOLYSACCHARIDE AND CARBOHYDRATE METABOLISM (*40, 45*)

Connective tissue, such as constitutes the bulk of the dermis, functions not only as a supporting medium but also has vital roles in many physiologic processes because it serves as the pathway for exchange of substances between the circulation and parenchymal cells. The extracellular portions of connective tissue are composed of fibers embedded in a complex amorphous ground substance. The fibers as well as many other constituents of the ground substance appear to originate from the metabolic activities of fibrocytes.

Collagen and elastin are the fibrous components of connective tissue. They are tough, elastic protein fibers, insoluble in organic solvents, cold water, dilute acids, alkalies, and salt solutions. When suitably tanned the connective tissues containing these fibers constitute leather. Collagen becomes solubilized in boiling water to yield gelatin.

Peptic enzymes digest native collagen whereas trypsin does not. In contrast to keratins, the chemical composition and physical properties of collagen from various mammalian species is remarkably uniform. As concerns its amino acid composition, collagen has a high content of glycine, proline, and hydroxyproline, a very low content of methionine, tyrosine, and histidine, and lacks cystine and tryptophane. The hydrolysates also uniquely contain the amino acid, hydroxylysine (*46*). Cross-linking of the peptide chains in collagen fibers consists only of hydrogen bonds and salt linkages. The special amide-hydroxyl type of hydrogen bond link is especially prevalent in collagen. Disulfide bonds so numerous in keratin seem to be entirely lacking. The polar side chains forming the salt linkages are long and account for the relatively "open structure" of collagen fibers and thus for their much greater hydration when compared with keratin. Native collagen contains about 66 percent water.

Electron microscopy reveals that collagen fibers at the macromolecular level are composed of very thin fibrils which when dry show cross-striations with repeating dark bands about 644 A apart (*47*). X-ray diffraction studies of collagen indicate a pattern generally differing from the pattern found in the keratin-myosin-fibrinogen group of fibrous proteins. The absence of well-defined lateral spacings suggests that the protofibril of collagen, in contrast to keratin, has the width only of a very few polypeptide chains. At the level of dark cross-striations, salt linkages are thought to prevail and swelling of the fibrillae in solutions of acids and bases occurs primarily at these points. This marked swelling of collagen and gelatin in dilute acids, bases, and neutral salts by combining with them is a distinguishing important property. Various anions and cations can be arranged in a so-called "lyotropic or Hofmeister series" according to their ability to cause such swelling (*48*). Thiocyanate, iodide, and bromide among anions and lithium, sodium, and potassium among cations are high in this list.

Elastin is much less abundant in skin than collagen and much less is known about it. It is more resistant than collagen to dilute acids and alkalies and to boiling water. Pepsin as well as trypsin attack elastin very poorly though a special pancreatic enzyme "elastase" does solubilize it without liberating amino acids. Elastin appears to contain less arginine, lysine, hydroxyproline, glutamic acid, and aspartic acid than collagen, but has more valine. Elastin has tougher rubber-like elastic properties than collagen and swells poorly. No cross-striations are evident on electron microscopy and the X-ray pattern is an amorphous one. These features suggest that elastin molecules lie in a randomly crumpled position and have relatively few but very strong cross-links (*49*).

From purely anatomic considerations the ground substance matrix in which these fibers are embedded must contain a large variety of substances and metabolites in transit between the blood stream and cells. In addition to these transit compounds and soluble protein precursors of fibers, the ground substance is characteristically rich in materials known as acid mucopolysaccharides. Although much remains to be discovered about these substances, increasing investigative effort in recent years has yielded important information about them. These compounds are linear polyelectrolytes of high molecular weight composed of repeating disaccharide units that contain an acetylated hexosamine and a uronic acid. In some cases a sulfate group is present probably on the hexosamine unit. Hyaluronic acid and several chondroitin sulfuric acids are the major dermal mucopolysaccharides. Tracer studies have shown that there is a very rapid metabolic turnover of these substances. The half life of hyaluronic acid in rodent skin is around 3.5 days and that of chondroitin sulfuric acid about 8.5 days (*50, 51*). The presence of both carboxyl and sulfate groups in the latter on each repeating disaccharide subunit confers a high negative charge to the chondroitin sulfuric acid. Such highly charged linear polyelectrolytes interact with water and cations such as sodium. Thus these substances probably serve important roles in regulating hydration and electrolyte concentrations about cells and in basement membranes. The biosynthesis of the mucopolysaccharides is believed to originate by the action of enzymes on a series of uridine nucleotides that contain their characteristic monosaccharide components. This type of biosynthesis is rather analogous to the manner in which uridine diphosphoglucose is used to build glycogen by the action of UDPG transferase.

As concerns carbohydrate metabolism in the skin, glycogen has received particularly much attention. It is especially abundant in fetal skin and is present in the epidermis, hair follicles, and sweat glands. Relatively on a per gram basis, the glycogen content of skin is about 1/150 that of liver. Adachi, however, has recently found evidence suggesting that the rate of turnover of cutaneous glycogen is very rapid and exceeds that in the liver (*51*). Cutaneous glycogen seems to be involved in the keratinization process and in sweat production as conspicuous changes in glycogen content of cells accompanies these processes.

Two peculiarities of cutaneous carbohydrate metabolism worth noting are 1) that although the Krebs tricarboxylic acid cycle operates in skin (*52*), unusually large amounts of succinic acid are produced and accumulate as recently shown by Halprin and Chow (*53*), and 2) that the epidermis is very active in forming lactic acid from glucose not only anaerobically, but also aerobically (*54*) though in this respect it is not quite as active as malignant tissues.

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SKIN CANCERS IN JAPAN: A NATIONWIDE 5-YEAR SURVEY, 1956-1960[1]

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SUMMARY

This paper reviews a 5-year (1956–60) survey of 851,685 first visits of patients to dermatological clinics in 41 Japanese medical university hospitals. The survey showed 921 cutaneous carcinomas, 81 malignant melanomas, 62 cutaneous sarcomas, and 95 malignant tumors of hematopoietic and lymphatic organs, compared to 3,270 diagnoses of psoriasis vulgaris and 3,689 of lupus erythematodes. Cutaneous carcinoma occurred in males 60 to 64 years of age and in females 55 to 59. Cutaneous sarcomas showed a level age distribution except for low incidences at 10 to 14, 25 to 29, and above 75 years of age. Sites of primary tumors, the duration, and size of the tumor at the time of the patients' first visits as well as possible precancerous lesions were analyzed. Among various preceding diseases, trauma and burns were important, especially on the scalp. Ten to 16 percent of the patients with precancerous dermatosis, xeroderma pigmentosum, chronic ulcer, and radiodermatitis showed malignant changes at the time of their first visits. Higher incidence of malignant tumors was observed in the southern and western areas than in the northern and eastern. This geographic distribution simulated that of lupus erythematodes and contrasted with that of psoriasis vulgaris.—NCI Monogr. No. 10: 55–70, 1963.

IN COLLABORATION with departments of dermatology in 41 of 46 Japanese medical school hospitals, we made a nationwide survey of various types of skin tumors and reviewed available histologic sections of biopsies. This survey was representative of the majority, if not all, skin cancers in Japan, except for patients diagnosed, treated, and followed by private practitioners or at hospitals not affiliated with medical schools. Detailed clinical histories of preceding diseases, the onset, duration, site, and appearance of the lesion at the first visit of the patient, and histologic diagnosis of malignant and premalignant skin lesions were analyzed. At the same time, surveys on psoriasis vulgaris and lupus erythematodes were made to compare the geographical difference of incidence with that of skin tumors. Clinical records of 851,684 first visits of patients to dermatological clinics of university hospitals are shown in table 1.

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

TABLE 1.—Total number of new visits to dermatological clinics at 41 university hospitals during 1956–60

Year	New visits
1956	159, 580
1957	162, 535
1958	168, 855
1959	176, 858
1960	183, 857
Total	851, 685

INCIDENCES OF SKIN TUMORS AMONG DERMATOLOGY PATIENTS

Among 851,685 new visits of patients to dermatological clinics during 1956–60 inclusive, 921 cutaneous carcinomas, 81 malignant melanomas, 62 cutaneous sarcomas including 9 cases of Kaposi's sarcoma, and 95 malignant tumors of hematopoietic and lymphatic organs were noted. There were also 3,270 patients with psoriasis vulgaris and 3,689 with lupus erythematodes during the same period.

These skin tumors were further divided according to clinical and histologic features as shown in table 2. Excluding metastatic carcinomas, of 921 patients with cutaneous carcinomas there were 855 with carcinoma and related lesions, an incidence of 0.10 percent. Comparable figures have been reported in Japan by Kitamura (*1*) who found 48 cases among 42,342 patients (0.11%) at Tokyo University during 1946–51, by Homma (*2*) who observed 24 cases among 22,053 patients (0.11%) at Gunma

TABLE 2.—Number of malignant and premalignant cutaneous lesions

Lesions	Male	Female	Total
Epidermoid carcinoma	317	224	541
Basal-cell carcinoma	106	94	200
Paget's disease	27	35	62
Bowen's disease	24	20	44
Erythroplasia	4	4	8
Metastatic carcinoma	38	28	66
Melanosarcoma	44	37	81
Cutaneous sarcoma	32	30	62
Malignant lymphoma	44	17	61
Leukemia	20	14	34
Mycosis fungoides	23	7	30
Xeroderma pigmentosum	70	84	154
Radiodermatitis	147	219	366
Chronic ulcer	71	63	134
Leukoplakia	8	8	16
Senile keratosis	31	17	48
Epidermodysplasia verruciformis	17	13	30
Keratoacanthoma	6	3	9
"Precancerous dermatosis"	7	9	16
Sarcoid	34	51	85
Recklinghausen's disease	223	302	525
Acanthosis nigricans	40	28	68

University during 1945–46, by Aiko *et al.* (*3*) who found 30 cases among 25,681 patients (0.12%) at Kagoshima University during 1951–59, and by Hosokawa (*4*) noting 35 cases among 22,355 patients (0.16%) at Hiroshima University during 1949–60. Miki (*5*) found similar incidence figures at Osaka University during 1957–59 and compared them to the much higher figures he found at the University of Colorado during 1956–59 (table 3).

TABLE 3.—Incidence of skin cancer: Comparison between University of Colorado and Osaka University*

		Colorado University			Osaka University		
Year		1956	1957	1958	1957	1958	1959
New visits of dermatological patients		594	652	601	9, 610	10, 399	9, 356
Skin cancer	Number	91	107	105	11	15	9
	Percent	15. 3	16. 4	17. 5	0. 1	0. 1	0. 1

*MIKI, Y.: Skin Res. 2: 23–30, 1960 (*5*).

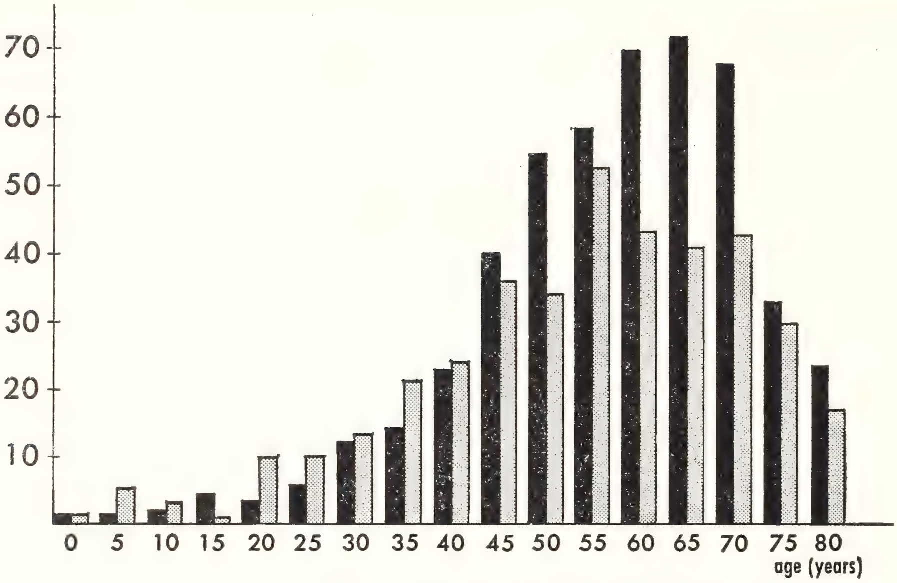
It was reported that the ratio of incidence of skin cancer among Caucasians and Japanese in Hawaii was 40 to 1. Nosko *et al.* (*6*) also reported that the incidence of 2,652 cases of skin cancer among new patients at the University of Vienna from 1934–53 was 2.1 percent. We expected to find in the present study a lower incidence in Japan than was found at the University of Vienna by the same sort of observation.

AGE AND SEX

The age and sex of the patients with cutaneous carcinoma in general, including epidermoid, basal-cell carcinoma, Paget's and Bowen's disease, and metastatic carcinoma, are shown in text-figure 1. When the age and sex distributions of squamous-cell carcinoma, basal-cell carcinoma, and malignant melanoma were observed separately, the curves shown in text-figure 2 were obtained. Both squamous-cell and basal-cell carcinomas most frequently occurred between ages 60 and 70. Twice as many males as females had squamous-cell carcinoma, but no appreciable difference in sex was noted in basal-cell carcinoma. Because of the majority of males among dermatological patients, basal-cell carcinoma was considered to be preponderant among females. Malignant melanoma showed a level age distribution with no apparent sex difference.

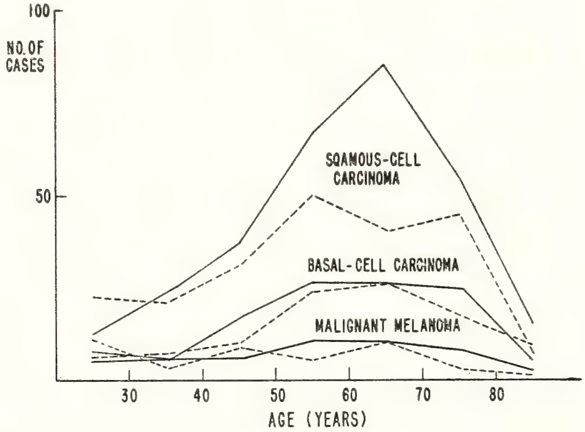
SITE OF PRIMARY LESIONS

In Japan, the scalp and face were frequent sites of primary lesions in both squamous-cell and basal-cell carcinomas as shown in text-figures 3 and 4. The ear lobes, lips, and dorsum of hands were not frequent sites for squamous-cell carcinoma. That there were only 48 senile keratoses



♂	1	1	2	4	3	6	12	14	23	40	55	59	71	73	69	33	24
♀	1	5	3	1	10	10	13	21	24	36	34	53	44	41	43	30	17

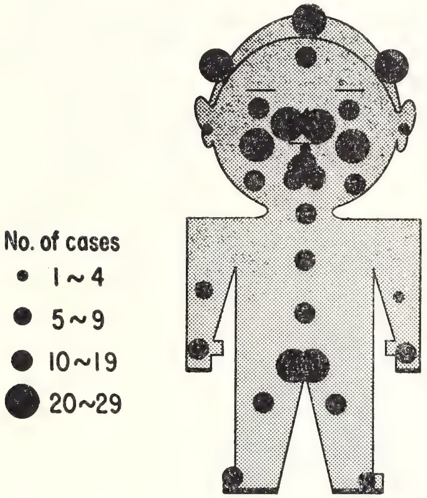
Text-figure 1.—General distribution by age and sex of patients with cutaneous carcinoma.



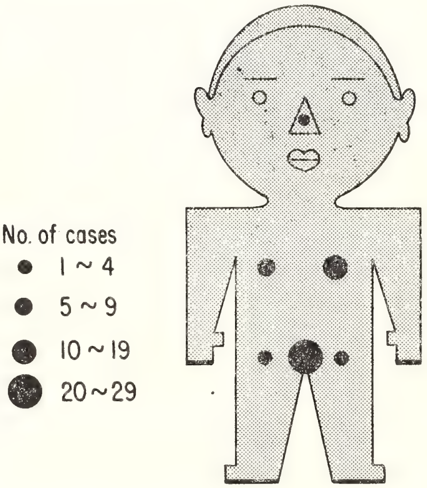
Text-figure 2.—Age and sex distribution of patients with squamous-cell and basal-cell carcinoma and malignant melanoma.

in over 850,000 dermatology patients, that the incidence of atrophic, precancerous skin of farmers and sailors among Japanese except for xeroderma pigmentosum is quite low, and that the scalp was the site of trauma and burns which had preceded many cases of skin cancer in Japan might explain this peculiar distribution.

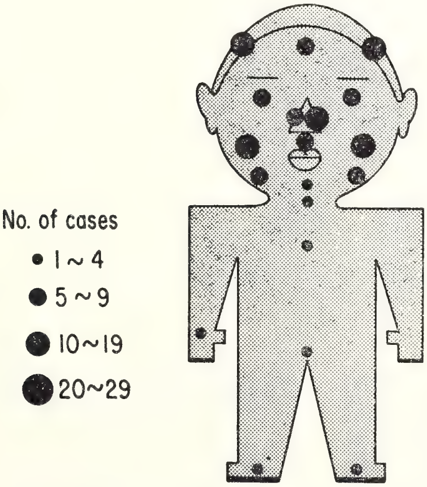
Fifty-one patients with histologically confirmed Paget's disease showed a high incidence in the area of the genitalia as shown in text-figure 5. On the other hand, in 35 patients with histologically proved Bowen's disease there was a more even distribution over the body as shown in text-figure 6. Malignant melanoma, cutaneous sarcomas, and malignant tumors of hematopoietic and lymphatic organs were rather evenly distributed, though malignant melanoma was more or less densely localized in the trunk and malignant tumors in the neck and trunk (text-figs. 7, 8, and 9).



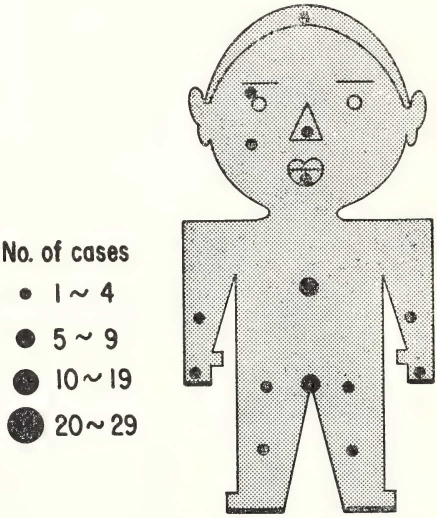
Text-figure 3.—Squamous-cell carcinoma of the scalp and face (541 cases).



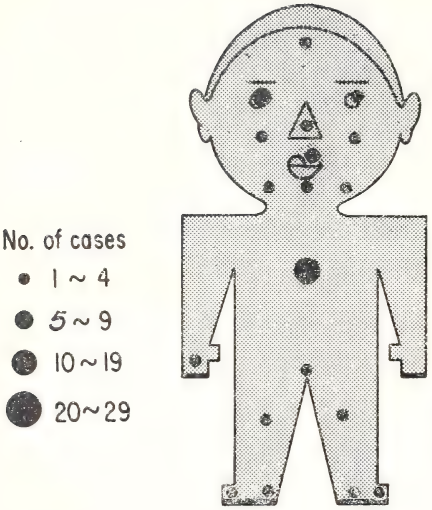
Text-figure 5.—Incidence of Paget's disease (51 cases).



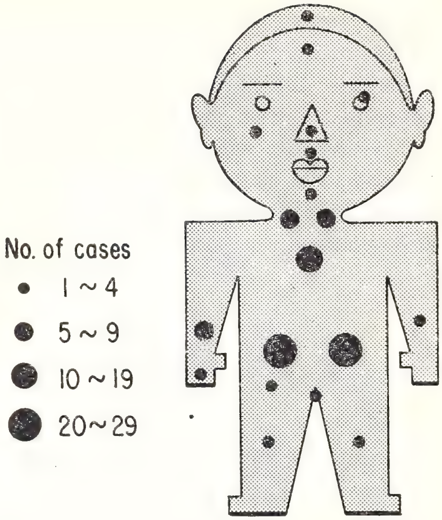
Text-figure 4.—Basal-cell carcinoma of the scalp and face (200 cases).



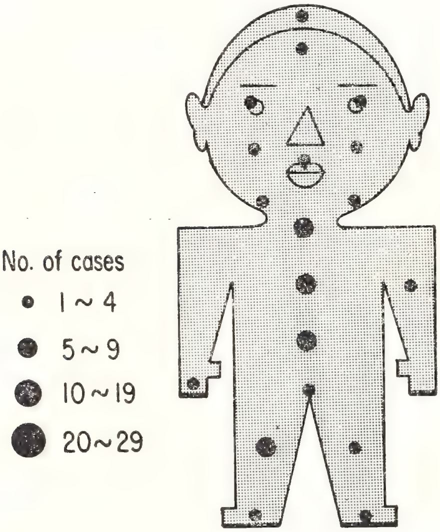
Text-figure 6.—Distribution of Bowen's disease (35 cases).



Text-figure 7.—Distribution of malignant melanoma (81 cases).



Text-figure 8.—Distribution of malignant tumors of the hematopoietic and lymphatic organs (110 cases).

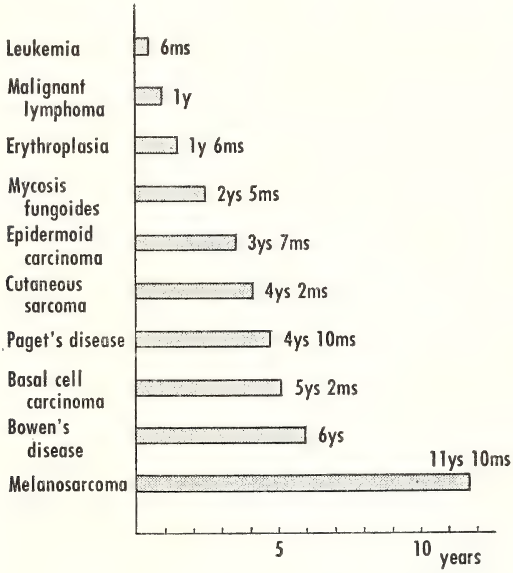


Text-figure 9.—Distribution of cutaneous sarcoma (60 cases.)

The mean tumor duration before the first visit was calculated on the basis of the period from the time the patient noticed the skin lesion until the first visit to the clinic. Consequently the duration could not always be measured accurately and is sometimes vague and longer than expected, as shown in text-figure 10. For example, 11 years and 10 months as the mean duration of malignant melanoma seemed very long, and we may wonder whether the patient had noticed a benign preceding lesion as the first appearance of malignancy. This long duration of skin tumors might also be explained by the delay of patients in visiting the clinics. Perhaps they waited until the tumor had grown or until they were advised to have medical attention. This procrastination is indicated by the size of the lesions at the patients' first visits to the clinics (table 4).

TABLE 4.—Size of skin cancer at the time first seen

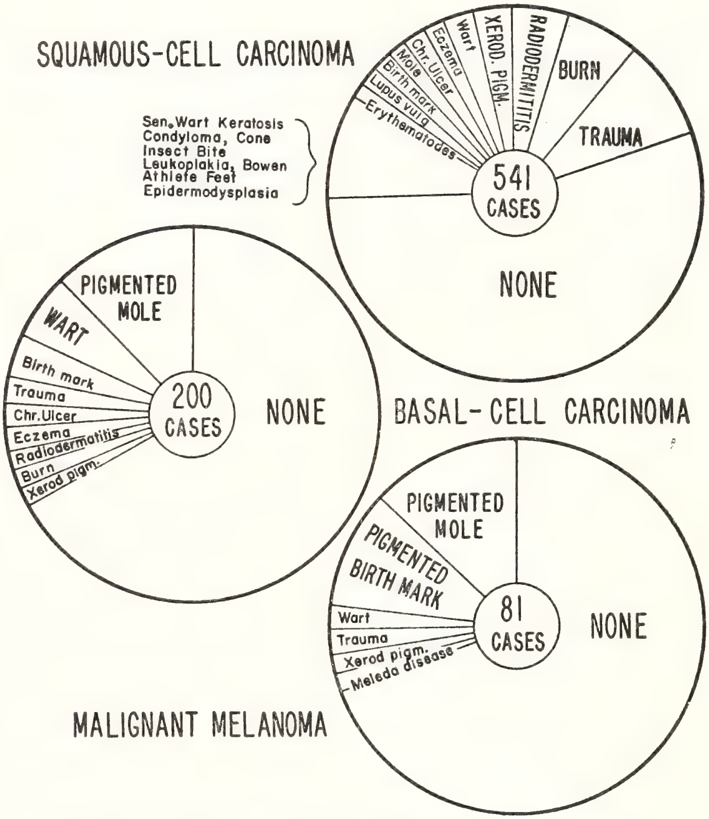
	Squamous-cell carcinoma		Basal-cell carcinoma		Malignant melanoma	
Size (cm)	Number of cases	Cumulative (%)	Number of cases	Cumulative (%)	Number of cases	Cumulative (%)
$\leqq 1$	52	20	52	39	10	21
$1 < \leqq 3$	98	58	58	83	24	72
$3 < \leqq 5$	73	86	13	93	8	89
< 5	36	100	10	100	5	100
Unknown	282	—	67	—	34	—
Total	541	—	200	—	81	—



TEXT-FIGURE 10.—Mean duration of tumor before the first visit.

PRECEDING DISEASES OF SKIN CANCERS AND PRECANCEROUS LESIONS

As much as 22.5 to 48.6 percent of skin malignancies were preceded by other dermatoses as shown in text-figures 11 and 12. Trauma and burn often occurred before squamous-cell carcinoma and pigmented mole previous to basal-cell carcinoma. But the latter lesion was almost invariably pigmented to a degree that led to an erroneous diganosis of malignant melanoma. Therefore, a pigmented mole may have been a quiescent basal-cell carcinoma. The same might be true of a malignant melanoma.

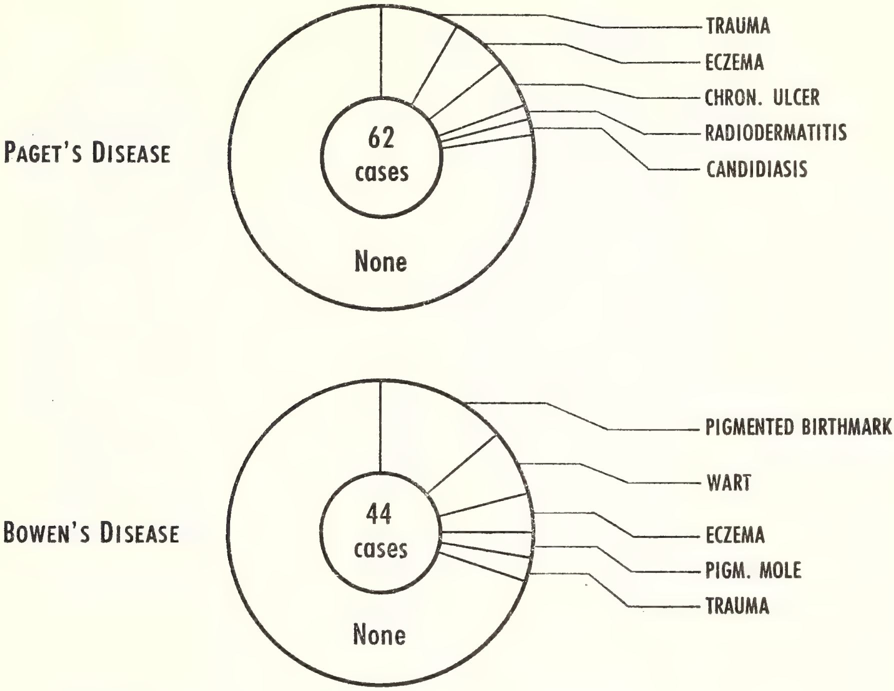


TEXT-FIGURE 11.—Preceding diseases in skin cancers.

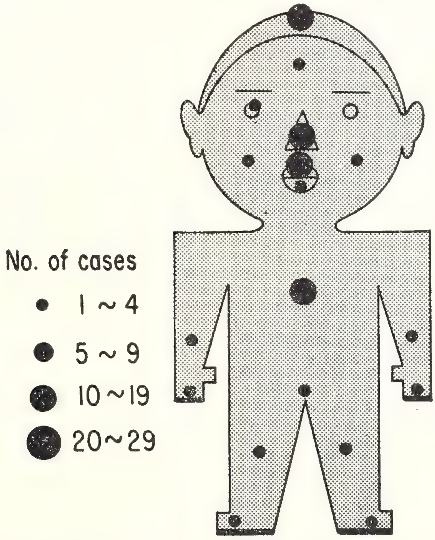
Sites of squamous-cell carcinoma of the skin are illustrated in text-figures 13, 14, 15, 16, and 17.

The mean duration of preceding diseases before malignant changes ranged from 2 years and 9 months for senile wart up to 33 years and 2 months for pigmented mole, as shown in text-figure 18. Age and sex distribution of these precancerous dermatoses is shown in table 5. It is interesting that several patients with xeroderma pigmentosum were in the age group over 40.

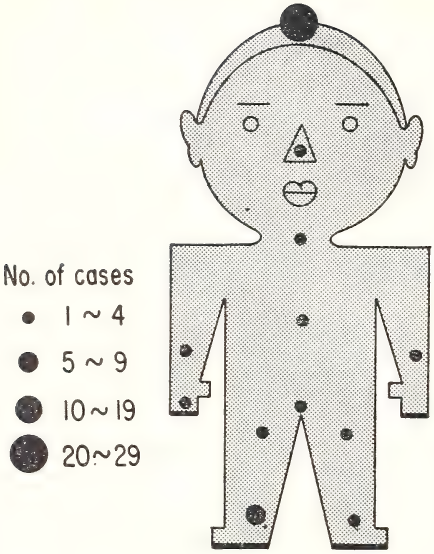
The incidence of malignant changes in precancerous dermatoses was taken from the number of patients in which the lesion in, or adjacent to, dermatoses was diagnosed as malignant at the first visit; almost all were histologically confirmed (text-fig. 19).



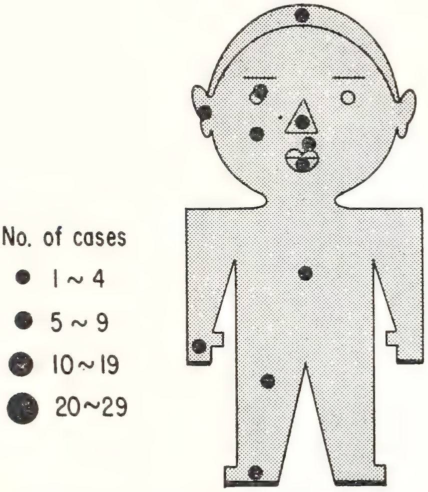
TEXT-FIGURE 12.—Preceding diseases in Paget's and Bowen's diseases.


No. of cases
● 1 ~ 4
● 5 ~ 9
● 10 ~ 19
● 20 ~ 29

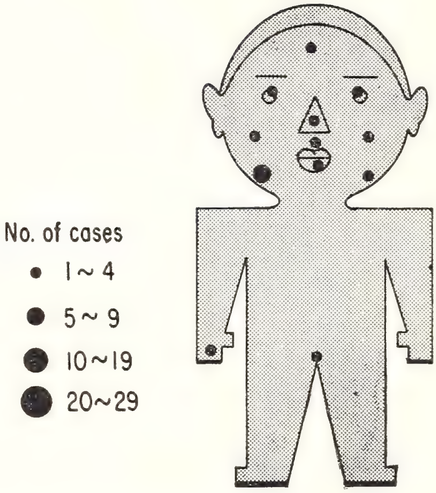
TEXT-FIGURE 13.—Fifty-three cases of skin cancer following trauma.


No. of cases
1 ~ 4
5 ~ 9
10 ~ 19
20 ~ 29

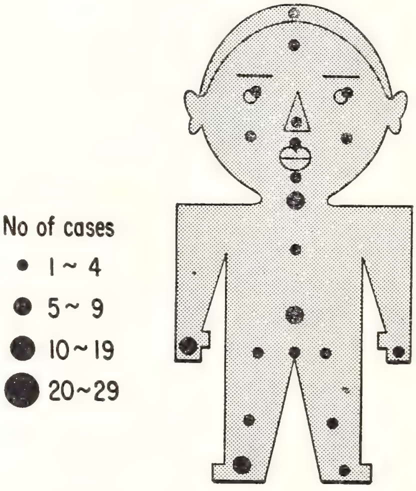
TEXT-FIGURE 14.—Forty-two cases of skin cancer following a burn.


No. of cases
1 ~ 4
5 ~ 9
10 ~ 19
20 ~ 29

TEXT-FIGURE 15.—Fifteen cases of skin cancer following chronic ulcer.


No. of cases
1 ~ 4
5 ~ 9
10 ~ 19
20 ~ 29

TEXT-FIGURE 16.—Twenty-five cases of skin cancer following xeroderma pigmentosum.

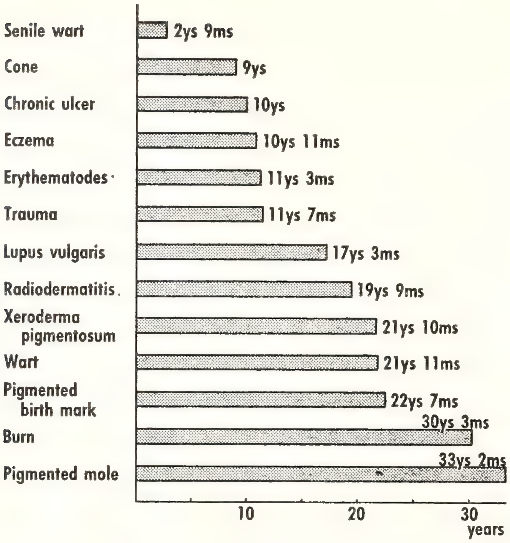

No of cases
1 ~ 4
5 ~ 9
10 ~ 19
20 ~ 29

TEXT-FIGURE 17.—Forty-three cases of skin cancer following radiodermatitis.

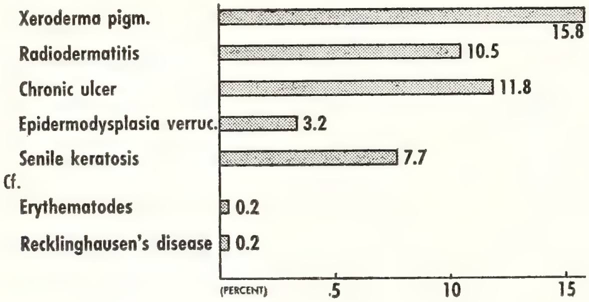
The incidences were: 15.8 percent for xeroderma pigmentosum; 11.8 percent, chronic ulcer; and 10.5 percent, radiodermatitis. Patients with psoriasis vulgaris and lupus erythematodes, who were surveyed as controls, had malignant change in only 0.2 percent.

Table 5.—Precancerous dermatoses, age and sex distribution

Disease	Sex	Age (yr) 0 ~	5 ~	10 ~	15 ~	20 ~	25 ~	30 ~	35 ~	40 ~	45 ~	50 ~	55 ~	60 ~	65 ~	70 ~	75 ~	80 ~
Xeroderma pigmentosum	♂	15	17	2	9	10	3	2	2	2	2	1		1			1	
	♀	22	26	4	11	6	3	2	2	1	3	1						
Radiodermatitis	♂	5	4	4	3	5	9	7	13	11	17	19	18	14	10	4	1	2
	♀	13	5	6	13	18	13	18	13	10	28	21	27	12	4	4	2	1
Chronic ulcer	♂	1		2	5	8	9	5	5	6	7	2	5	4	5	3	2	1
	♀	2	2	1	8	10	5	2	13	2	4	3	3	4	1			
Leukoplakia	♂								1		1	3	1	1				
	♀		1					1			3		2	1				
Senile keratosis	♂					1				2	4	4	7	6	4	3		
	♀								3		2	3		5	2	1		
Epidermodysplasia verruciformis	♂				3	6		2	1		2			1	1		1	
	♀	2	1	2		3	1	1	2	1								
Keratoacanthoma	♂							2		1		1	1			1		
	♀											2			1			
"Precancerous dermatosis"	♂	1									2	1	1			2		
	♀				1		1				1	1	1	1	1	1	1	



TEXT-FIGURE 18.—Mean duration of preceding diseases before apparent malignant changes.



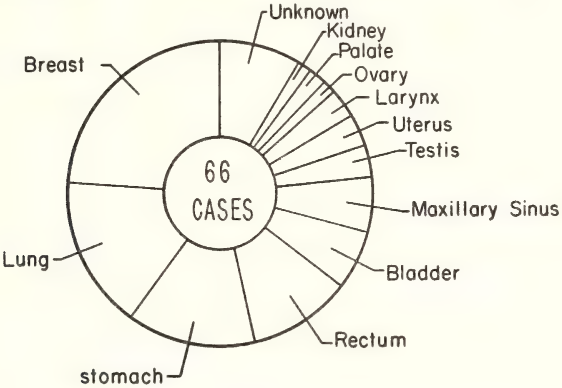
TEXT-FIGURE 19.—Incidence of malignant changes in precancerous dermatoses.

METASTATIC CARCINOMA OF SKIN

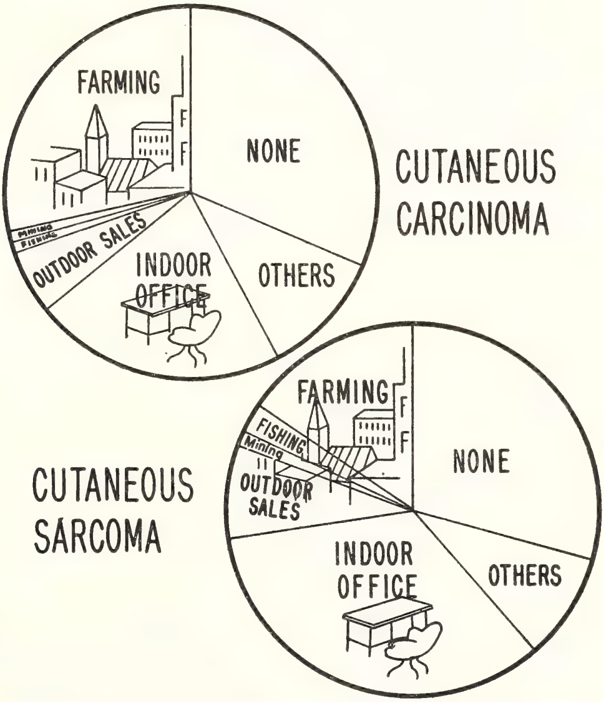
There were 66 patients with metastatic carcinoma of the skin; the primary lesion was noted in the breast in about 25 percent, and in lung, stomach, rectum, and bladder (text-fig. 20).

OCCUPATIONAL FACTORS IN SKIN CANCER

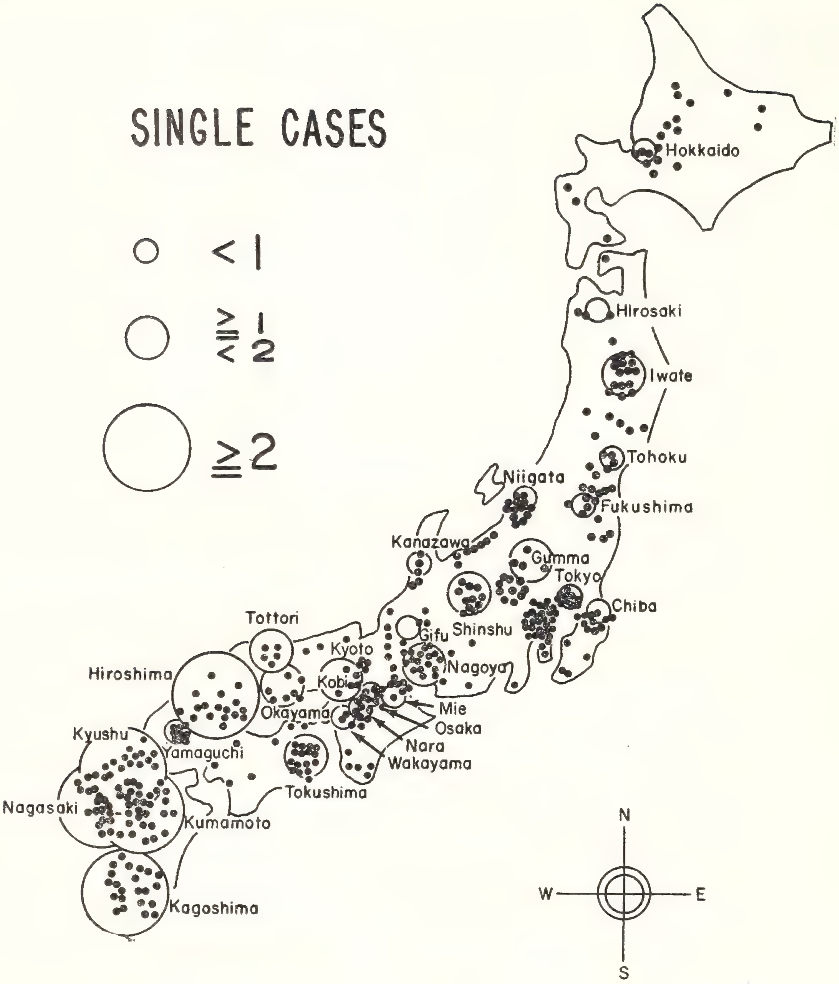
We could not compare detailed, classified, occupational factors concerning skin cancer and noncancer patients to elucidate any one factor involved in the development of skin cancer. However, there was some difference in the influence of sunlight when the incidences of carcinomas and sarcomas were compared, as shown in text-figure 21; *i.e.*, there were more patients, who had been exposed to sunlight, with carcinoma than sarcoma.



TEXT-FIGURE 20.—Primary lesion of metastatic carcinoma of skin.



TEXT-FIGURE 21.—Occupational factors in skin cancer.

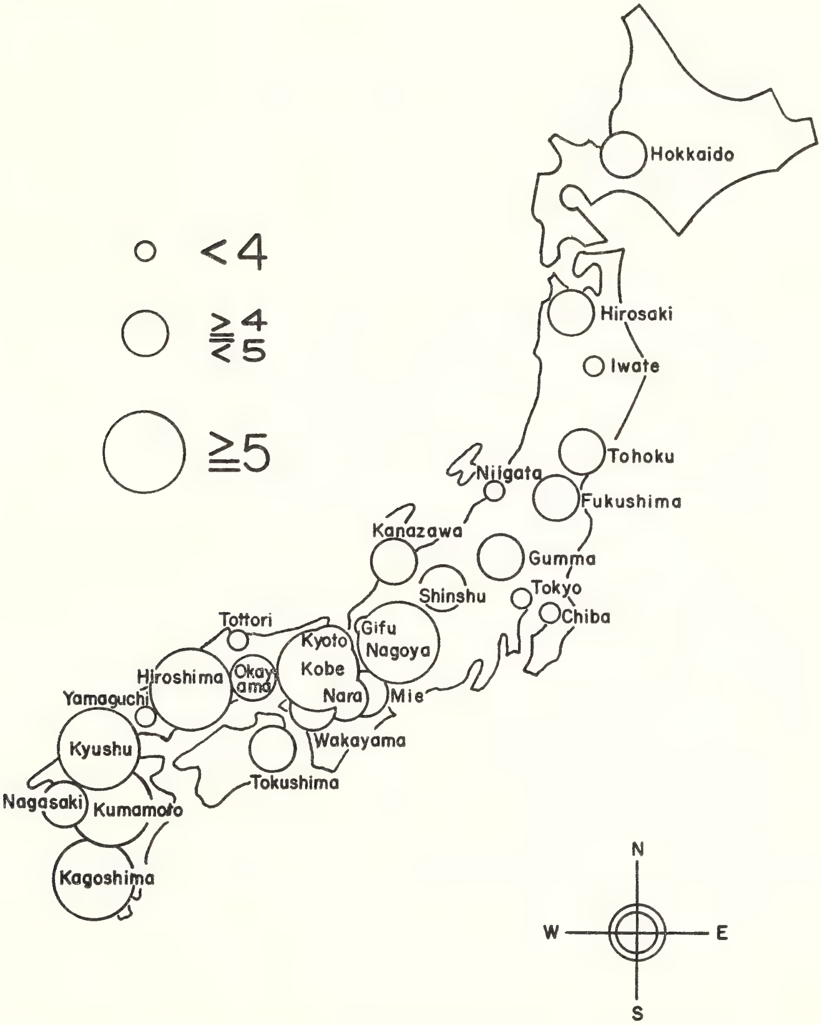

SINGLE CASES
< 1
≧ 1
< 2
≧ 2
Hokkaido
Hirosaki
Iwate
Tohoku
Niigata
Fukushima
Kanazawa
Gumma
Tokyo
Chiba
Tottori
Shinshu
Gifu
Kyoto
Nagoya
Hiroshima
Kobe
Okayama
Mie
Osaka
Nara
Wakayama
Kyushu
Yamaguchi
Tokushima
Nagasaki
Kumamoto
Kagoshima
N
W
E
S

Text-figure 22.—Number of skin cancers per 1,000 first visits of patients to dermatological clinics.

GEOGRAPHIC DISTRIBUTION OF SKIN CANCER AND ITS COMPARISON WITH PSORIASIS VULGARIS AND LUPUS ERYTHEMATOSUS

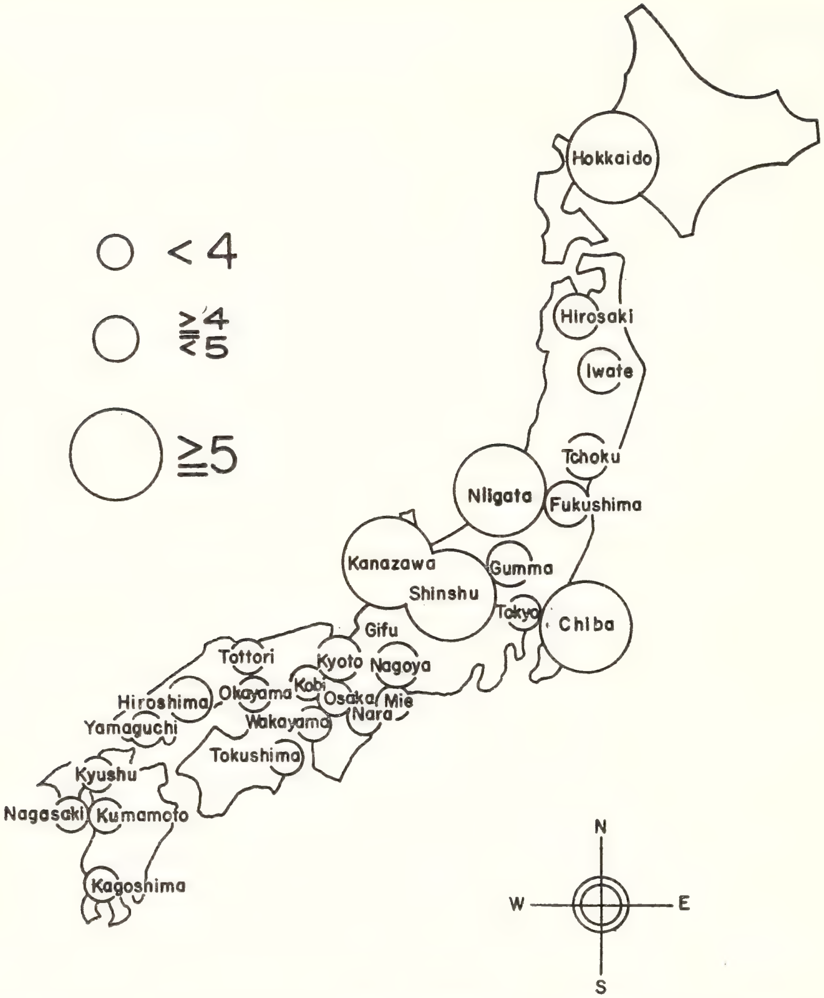
When a map of Japan was dotted for each case of skin cancer, aggregation of cases in large cities was observed as expected.

When the number of skin cancers per 1,000 new dermatological visits in each medical school hospital was compared, an interesting result was noted as shown in text-figure 22. This geographic distribution of skin cancer was similar to that of lupus erythematodes (text-fig. 23) and suggests that there was more sunlight in the southern and western areas than in the northern and eastern areas. Sunlight precipitates or aggravates lupus erythematodes.


<4
≧4
<5
≧5
Hokkaido
Hirosaki
Iwate
Tohoku
Niigata
Fukushima
Kanazawa
Gumma
Shinshu
Tokyo
Chiba
Tottori
Gifu
Nagoya
Kyoto
Kobe
Okayama
Hiroshima
Yamaguchi
Nara
Mie
Wakayama
Kyushu
Tokushima
Nagasaki
Kumamoto
Kagoshima
N
W
E
S

TEXT-FIGURE 23.—Number with lupus erythematosus per 1,000 first visits of patients to dermatological clinics.

When the distribution of psoriasis vulgaris was similarly mapped (text-fig. 24), the opposite result was obtained. The higher incidence was in the northern areas. This agreed with the idea that sunlight is beneficial for psoriasis.


< 4
≧4
<5
≧5
Hokkaido
Hirosaki
Iwate
Tchoku
Niigata
Fukushima
Kanazawa
Gumma
Shinshu
Tokyo
Chiba
Gifu
Tottori
Kyoto
Nagoya
Kobe
Okayama
Osaka
Mie
Nara
Hiroshima
Yamaguchi
Wakayama
Tokushima
Kyushu
Nagasaki
Kumamoto
Kagoshima
N
W
E
S

TEXT-FIGURE 24.—Number with psoriasis vulgaris per 1,000 first visits of patients to dermatological clinics.

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REGIONAL INCIDENCE AND PATHOLOGY OF SKIN CANCER IN THAILAND[1]

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SUMMARY

A study of surgical specimens at the laboratories of the Siriraj Hospital, Bangkok, 1949–60, revealed 11,080 cases of cancer, including 1,020 skin cancers. Skin cancer occurred more commonly in Thai than in Chinese (ratio 9 : 1), and in males than in females (ratio 3 : 2). Squamous-cell carcinoma comprised more than 70 percent of all skin cancers and occurred most commonly on the head (30.3%), penis (25.6%), legs (13.6%), and vulva (11.2%). Exposure to sunlight, chronic ulceration, trauma, and poor hygiene appear to be the most likely predisposing factors.—NCI Monogr. No. 10: 71–74, 1963.

SKIN CANCER ranks third among the specimens examined in the laboratories of the Siriraj Hospital, Bangkok (*1*). The material obtained in this analysis was collected from the files of the Departments of Pathology and Obstetrics and Gynecology of Siriraj Hospital Medical College for 12 years, 1949–60. The specimens included those sent from other hospitals within the area of Bangkok and also from several provincial hospitals throughout Thailand. The total number of cancer cases was 11,080, of which 1,020 were skin cancer, an incidence of 9.29 percent. Skin cancer has its highest incidence in the sixth decade, which is true for almost all cell types. Males had a higher incidence than females by a ratio of 3 : 2. Thai and Chinese patients showed a racial incidence of 9 : 1 (tables 1, 2, and 3).

Among 1,020 cases of skin cancer the following main histological types were distinguished:

Squamous-cell carcinoma	663
Basal-cell carcinoma	210
Malignant melanoma	55
Undifferentiated carcinoma	26
Basosquamous-cell carcinoma	5
Sebaceous carcinoma	8
Sweat-gland carcinoma	4
Mycosis fungoides	1
Dermatofibrosarcoma	2
Intraepidermal carcinoma	5
Senile keratosis	41

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

TABLE 1.—Sex and race incidence

Type	Sex		Race	
	Male	Female	Thai	Chinese
Squamous-cell carcinoma	442	221	595	68
Basal-cell carcinoma	111	99	190	20
Malignant melanoma	35	20	51	4
Undifferentiated carcinoma	9	17	23	3
Basosquamous-cell carcinoma	1	4	5	—
Sebaceous carcinoma	3	5	8	—
Sweat-gland carcinoma	3	1	3	1
Mycosis fungoides	1	—	1	—
Dermatofibrosarcoma	1	1	1	1
Intraepidermal carcinoma	1	4	5	—
Senile keratosis	23	18	37	4
Total	630	390	919	101

TABLE 2.—Age incidence

Type	10–20	21–30	31–40	41–50	51–60	61–70	71+	Not known	Total
Squamous-cell carcinoma	5	43	88	142	169	126	68	22	663
Basal-cell carcinoma	1	4	8	36	67	51	35	8	210
Malignant melanoma	—	5	10	9	16	11	3	1	55
Undifferentiated carcinoma	—	2	1	5	7	2	5	4	26
Basosquamous-cell carcinoma	—	—	—	—	2	2	1	—	5
Sebaceous carcinoma	—	1	—	2	3	—	2	—	8
Sweat-gland carcinoma	—	—	2	1	—	1	—	—	4
Mycosis fungoides	—	—	—	—	—	1	—	—	1
Dermatofibrosarcoma	—	1	—	—	1	—	—	—	2
Intraepidermal carcinoma	—	—	—	1	2	—	1	1	5
Senile keratosis	—	—	1	7	16	10	6	1	41
Total	6	56	110	203	283	204	121	37	1020

Table 3.—Body distribution

Type	Head and neck	Upper limb	Lower limb	Trunk	Penis	Vulva	Not mentioned
Squamous-cell carcinoma	201	42	90	74	170	75	11
Basal-cell carcinoma	187	1	2	16	—	—	4
Malignant melanoma	17	10	15	11	—	1	1
Undifferentiated carcinoma	6	2	5	11	2	—	—
Basosquamous-cell carcinoma	4	—	—	1	—	—	—
Sebaceous carcinoma	7	—	—	—	—	1	—
Sweat-gland carcinoma	—	2	1	—	—	—	1
Mycosis fungoides	—	1	—	—	—	—	—
Dermatofibrosarcoma	—	—	1	1	—	—	—
Intraepidermal carcinoma	1	1	—	3	—	—	—
Senile keratosis	18	5	5	6	1	—	6
Total	441	64	119	123	173	77	23
Percent	43. 2	6. 3	11. 6	12. 0	16. 9	7. 5	2. 2

SQUAMOUS-CELL CARCINOMA

The highest frequency of cancer of the skin was squamous-cell carcinoma. Squamous-cell carcinoma, intraepidermal carcinoma, senile keratosis, and undifferentiated carcinoma comprised 74.5 percent of the total skin cancers. Squamous-cell carcinoma in this series had a predisposition for the head (30.3%), penis (25.6%), lower limb (13.6%), and vulva (11.2%). Five cases of squamous-cell carcinoma were seen in the second decade, the youngest at age 15.

Carcinoma arising from old burn scars was noted in only 3 cases. Cancer arising from scars of vaccination against smallpox was never seen. Chronic ulcerations and infections of the skin of the foot from 1 to 10 years' duration were usually followed by carcinomatous changes. These occurred in farmers, gardeners, and workers who go barefooted.

Parts of the skin which are mostly exposed to sunlight such as the head and neck show some predisposition to the development of cancer. About one third of the squamous-cell carcinomas and senile keratoses were seen on the head and neck regions.

All cancers of the penis were of squamous-cell type and prevalent in patients living under poor economical conditions and with poor personal hygiene, such as farmers and coolies. Over 90 percent gave a history of phimosis. Only a few patients had clinical symptoms or history of yaws.

Of 77 cases of carcinoma of vulva, 75 were of squamous-cell type, 1 was a malignant melanoma, and 1 a sebaceous carcinoma. Carcinoma of the vulva was seen mostly in women past middle age with poor personal hygiene. Some patients admitted to a history of venereal diseases; the youngest was 30 years old.

Only 3 cases of arsenical carcinoma were recorded, none on the thorax.

BASAL-CELL CARCINOMA

Basal-cell carcinoma, forming about 20 percent of the total cases, was the next most common skin cancer. The highest incidence was in the sixth decade. The youngest patient seen was a 19-year-old Thai male with a lesion on the chest. Similar to squamous-cell carcinoma, basal-cell carcinoma was most prevalent on the skin of face, nose, and ears. Several tumors were pigmented and most of these were clinically diagnosed as melanoma. Five cases were diagnosed basosquamous-cell carcinoma, mostly in people over age 60.

MALIGNANT MELANOMA

There were 55 cases of malignant melanoma in this series, approximately 5 percent of all skin cancers. The distribution was as follows:

Head and neck	17
Upper limb	10
Lower limb	15
Trunk	11
Vulva	1
Not specified	1

Of 15 cases of malignant melanoma occurring on the lower extremities, 9 were on the sole of the foot. These 9 patients were mostly outdoor workers and usually they go barefooted during the course of their daily activities; the youngest was 30 years old.

Other types of skin cancer, *i.e.*, sebaceous carcinoma, sweat-gland carcinoma, dermatofibrosarcoma, and mycosis fungoides occurred in small numbers.

In consideration of the etiology of cancer of the skin, it appears that the development of skin cancer depends on the cell type, site of the tumor, exposure to sunlight, chance of being traumatized, degree of infection, and personal hygiene. Other factors such as: nature of occupation, burns, venereal diseases, yaws, arsenical preparations, native drugs, phimosis, collection of smegma, material from retained urine, and age of patient may play a part in the production of skin cancer.

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SOME ETIOPATHOLOGIC ASPECTS OF SKIN CANCER IN SOUTH VIETNAM[1]

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SUMMARY

The incidence, distribution, histologic types, and possible etiologic factors of skin cancer in South Vietnam are reviewed. Of 281 patients studied, skin cancer, particularly of the lip, was more common in women, with onset in the middle to older age group. Sunlight, alimentary deficiencies, and local disorders such as phimosis appeared etiologically significant. Histologically, squamous-cell carcinoma was found most frequently, while malignant melanoma was rare.—NCI Monogr. No. 10: 75–79, 1963.

FROM 1955 to 1960, 281 patients with skin cancer were treated at the Cancer Center of Binh Dan Hospital in Saigon and the Cancer Institute of South Vietnam. Of these, 56 (20%) had cancer of the exposed skin (excluding lip), 90 (32%) cancer of the lip, 87 (31%) cancer of the penis, and 48 (17%) cancer of the vulva. The sex distribution is shown in table 1. Particularly striking are the very high incidence of lip cancer in females and the prevalence of carcinoma of the penis over carcinoma of the vulva.

TABLE 1.—Sex distribution of skin cancer in South Vietnam

Sex	Exposed skin	Penis	Vulva	Lip	Total
Male	30	87	—	10	127
Female	26	—	48	80	154
Total	56	87	48	90	281

The mean ages at time of diagnosis are tabulated in table 2. In general, genital cancer arises earliest, cancer of the exposed skin slightly later, while cancer of the lip develops significantly later.

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

TABLE 2.—Age distribution of skin cancer in South Vietnam by site

Site	Male	Female	Minimum age (years)	Maximum age (years)
Exposed skin	52. 4	54. 5	10	79
Penis	47. 8	—	20	76
Vulva	—	50	22	77
Lip	67. 8	62	40	85

CANCER OF EXPOSED SKIN (EXCLUDING LIP)

In the 56 patients with cancer of the exposed skin, most lesions were found on the face and then, as shown in table 3, on the thighs, head, trunk, perineum, upper limbs, and neck.

TABLE 3.—Distribution of cancer of the exposed skin

Site	Number of lesions
Face	27
Thighs, legs	10
Head	8
Trunk	4
Perineum	3
Upper limbs	3
Neck	1

No obvious predisposing conditions were noted; only one tumor arose in a burn scar and one in a traumatic scar. Prolonged exposure to sunlight is the most likely contributory factor in this group, since 48.5 percent of the patients were laborers exposed to continuous tropical sun and 25.3 percent were outdoor workers. However, since only 20 percent of all lesions occurred on the sun-exposed areas, factors other than sunlight must be considered.

CANCER OF THE LIP

Cancer of the lip occurred primarily in women, particularly on either side of the midline of the lower lip (table 4). The fact that only 26 percent of the females were outdoor workers suggests that sunlight may not be the major etiologic factor (table 5). Tobacco chewing or smoking and a condition similar to Plummer-Vinson syndrome occurring in Vietnamese women may also be responsible.

TABLE 4.—Distribution of cancer of the lip by location and sex

Sex	Upper lip		Lower lip		Commissure	Multiple lesions
	Midline	Lateral	Midline	Lateral		
Male	—	1	—	8	—	—
Female	1	5	15	37	15	6
Total	1	6	15	45	15	6
	7		60			

TABLE 5.—Occupation of patients with cancer of the lip

Occupation	Males	Females
Indoor worker	—	30
Outdoor laborer	5	23
Trade worker	—	6
Clerk	1	2
Religious	—	1
No work	4	18

CANCER OF THE PENIS

Joyeux and Nguyen Van Nguyen reported an 8 percent incidence of cancer of the penis. In our statistical data, cancer of the penis occurs in 5.4 percent of all cancer in males, a figure significantly higher than that reported for most Western countries [1.25% (*1*)]. The disease occurs primarily among the poorer classes of the population. The major preexisting conditions were phimosis, which was noted in 90 percent of all patients, and the occasional presence of papillomatous lesions (1.5%). Metastasis to inguinal lymph nodes is common and was found in more than three quarters of the patients at the time of diagnosis of the tumor (*2–4*).

CANCER OF THE VULVA

Malignant tumors of the vulva are common in Vietnamese, and serious because of early lymph-node metastasis. Carcinoma of the vulva comprises 3.6 percent of all female genital cancer in our series as compared with 4.1 percent in that of Tran Dinh De (*5*), 5.5 percent in that of Joyeux, and 8 percent of that of Le Roy des Barres.

Of our patients, 43.7 percent were multiparous and 29 percent of these had more than 3 pregnancies, 43.7 percent were postmenopausal, and 22.9 percent had serologic evidence of syphilis (*6*). The distribution of carcinoma of the vulva is shown in table 6.

Table 6.—Distribution of carcinoma of the vulva

Location	Percent of cases
Labia majora	42. 1
Labia minora	23. 4
Vestibule	15. 9
Clitoris	10. 9
Periurethral	7. 7

HISTOLOGY OF SKIN CANCER

Microscopically, 55.5 percent of all lesions were squamous-cell carcinomas, 5.3 percent basal-cell carcinomas, and 7.1 percent intermediary carcinomas. A few other histologic types were noted (table 7). In comparison to other tropical areas, the rarity of malignant melanoma and of squamous-cell carcinoma in leg ulcers is noteworthy.

Table 7.—Histologic diagnosis of 281 patients with skin cancer in South Vietnam

Histologic type	Lip	Exposed skin	Penis	Vulva	Total
Squamous-cell carcinoma	57	33	43	23	156
Basal-cell carcinoma	1	8	1	5	15
Intermediate carcinoma	7	7	1	5	20
Undifferentiated carcinoma	—	—	1	6	7
Squamous-cell carcinoma (mucosa type)	—	—	—	1	1
Adenocarcinoma	—	—	1	2	3
Degenerated papilloma	—	—	5	—	5
Suspect papilloma	—	—	10	3	13
Inadequate biopsy	24	—	22	3	49
Cylindroma	—	1	—	—	1
Melanosarcoma	—	2	—	—	2
Malignant melanoma	1	—	1	—	2
Fibrosarcoma	—	2	—	—	2
Lymphosarcoma	—	1	1	—	2
Leukemia	—	1	—	—	1
Metastatic carcinoma	—	1	1	—	2
Total	90	56	87	48	281

DISCUSSION

In South Vietnam, cancer occurs on the exposed and unexposed parts of the skin. Genital tumors are common and clinically of the greatest importance.

The very high frequency of cancer of the lip in women is strikingly different from the incidence noted in other countries and cannot be explained by exposure to sunlight alone. Smoking, tobacco chewing, and alimentary deficiencies may be of etiologic importance.

Genital tumors in males are almost always preceded by phimosis, and in females may be associated with local disorders.

Histologically, squamous-cell carcinoma was most common, then degenerated papillomas, and basal-cell carcinoma. Malignant melanoma and other tumors were seen only rarely.

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RELATIVE INCIDENCE OF SKIN CANCER IN CHINESE IN TAIWAN: WITH SPECIAL REFERENCE TO ARSENICAL CANCER[1]

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SUMMARY

Skin cancer, including carcinomas of anus, penis, vulva, external auditory canal, and lip, malignant melanoma and sarcoma, is not uncommon in Chinese in Taiwan, but the incidence is by no means as high as that reported for Chinese on the mainland. Although the largest number of skin cancers falls in the age group 50–59, the age curve ratios of skin cancer to all patients examined in each decade shows a steady increase up to age 80. High incidence of penile cancer relative to other tumors has been reported from some parts in the mainland of China, but this is not the case in Taiwan. The frequency of malignant melanoma is not high in Taiwan. In the surgical series there were 22 cases definitely originating from skin (0.18% of all tumors), and in 4,000 autopsies there were only 3 cases of melanoma (0.08% of all autopsies and 0.52% of all tumors). It is of special interest that in a limited area on the southwest coast of Taiwan, with a population of approximately 160,000, a remarkably high percentage of chronic arsenical intoxication (owing to high concentration of arsenic in drinking water) has been observed in recent years. Up-to-date 64 arsenical cancers in 52 patients have been found in this area. Of these, 39 were intraepidermal carcinoma, 17 epidermoid carcinoma, and 8 basal-cell carcinoma. Multiple skin cancers were found in 12 patients. The pathology of arsenical melanosis, keratosis, and cancer is discussed and illustrated. In the same area an endemic peripheral vascular disorder resulting in gangrene of extremities, expecially the feet, has been noted. Clinically, its symptoms, signs, and course are similar to those of Buerger's disease. On examination of amputated legs, only one-fourth of the patients have vascular changes compatible with thromboangiitis obliterans while the remaining three-fourths show changes of arteriosclerosis obliterans. Of 225 such patients, 184 showed signs of chronic arsenical intoxication. Eighteen developed skin cancer. Of 94 saltern workers who had lived in this area for a period of years and moved at different ages to another saltern where the drinking water is not contaminated with arsenic, 68.08 percent showed arsenical melanosis, 21.27 percent keratosis, and 6.38 percent skin carcinoma. Evidently a majority of persons once exposed to arsenic intoxication for some time develop skin lesions including cancer many years later, even after drinking of contaminated water is discontinued.—NCI Monogr. No. 10: 81–107, 1963.

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

THE RELATIVE INCIDENCE of skin cancer in Chinese on the mainland of China as reported in the literature appears high, but the actual incidence is not known. Taiwan is an island, less than 100 miles east of the southeast coast of the mainland, with an area of 13,885 square miles and a population of 11.18 millions (1961). Geographically, it is located at latitude 21°45′25″ to 25°37′53″ north and longitude 119°8′13″ to 122°6′2″ east. The climate is subtropical, the highest registered temperature never above 38° C and the lowest not below 1° C. The average temperature is 21.6° C for Taipei in the north and 24.3° C for Kaohsiung in the south. There are 7,224 registered doctors (1961) engaged in medical practice, either as practitioners or serving in 35 public or government hospitals and in provincial and local health units including some 400 health stations. Taiwan is one of the Asian areas where medical service is accessible to the majority of the people except in remote and sparsely populated districts, but reliable health statistics are lacking. This paper deals with the relative incidence of skin cancer in Taiwan, based on records of surgical pathological examinations and autopsies which are much larger than any series previously reported from China. It is obvious that skin cancer, including carcinomas of anus, penis, vulva, external auditory canal, lip and other body surfaces, malignant melanoma and sarcoma, is not uncommon in Chinese in Taiwan, but the relative incidence is by no means as high as that reported in the past.

MATERIALS

The data used for statistical analysis consist of 100,554 surgical specimens and 4,000 autopsies. Of the former, 72,249 are consecutive surgical specimens submitted during 1946–61 to the Department of Pathology, National Taiwan University Hospital, which has 800 beds, the largest on the Island, and the remaining 28,314 are specimens examined at 9 other hospitals, 5 of which are located in southern parts and 4 in northern parts of Taiwan. In these 9 hospitals there was no pathology service until 1953, but since then a pathology laboratory has been created in each one. The figure 100,554 represents the number of surgical specimens examined, but not the number of patients. Actually there were many patients from whom biopsies were taken several times. Furthermore, in the 7 Army, Navy, Air Force Hospitals, and 1 Veteran Hospital the patients are predominantly males.

Of the 4,000 autopsies, 3,279 were performed in the Department of Pathology, National Taiwan University Hospital, of which 1,279 including 726 medicolegal cases were performed before 1946 under Japanese administration; the remaining were from the other hospitals mentioned. All the autopsies, prior to 1946, were done on Chinese inhabitants who had lived the greater portions of their lives in Taiwan or were born there. Those done after 1947 include the Chinese long resident in Taiwan and others moved in from the mainland.

Because skin cancers occur on the surface of the body and usually on the exposed surfaces, they are seen early by the patients and are easily accessible to pathological diagnosis and prompt treatment. Thus, the statistical analysis of such tumors should give a more accurate picture by use of surgical pathological data than by autopsy and mortality data. Although the curability is high and the mortality low, there were a number of cases which came to autopsy. There may also be incidental discovery of such tumors. Therefore, a statistical analysis of tumors on necropsies is also of some significance. The main purpose of this study is to report our data in sufficient detail so that comparisons and interpretation can later be made by others to ascertain whether etiological factors concerning the racial and geographical differences in skin cancer exist. In this series are included 52 cases of arsenical cancer of which 35 were discovered from a recent survey of chronic arsenic poisoning in 6 villages in a southern area of Taiwan where arsenic in excessive amount is found in drinking water from artesian wells. A special feature of this report is that not only arsenical cancer but related conditions are studied and discussed.

RELATIVE INCIDENCE OF SKIN CANCER BASED ON SURGICAL PATHOLOGICAL MATERIAL

Among 100,554 surgical specimens, including materials from major operations, excisions, biopsies, needle biopsies, puncture and tapping materials, and diagnostic smears, 12,084 were primary malignancies, 4,600 in males and 7,484 in females, and constitute 12.02 percent of all specimens examined histologically. The 20 most common varieties of malignant tumors, 96.15 percent of the total, are shown in table 1. Of the 12,084 malignant tumors, 282 or 2.33 percent were carcinomas of skin, occupying tenth position in the present data. There were 281 anatomically related tumors, *e.g.*, sarcomas, malignant melanomas, and carcinomas of the lip, penis, and vulva. If the 281 malignant tumors are added, there were 563 skin cancers, 4.65 percent of all malignant tumors, occupying sixth place. Tables 2 and 3 show the 16 most common malignant tumors in males and females, respectively. Of 4,600 malignancies in males there were 161 carcinomas of skin, 3.50 percent of the total and in eleventh place. If all other related malignancies are added, there were 325 *in toto*, 7.06 percent, in fifth place. Of the 7,484 malignancies in females, there were 121 carcinomas of skin (1.62%, ninth place). If other related malignancies of skin are added, there were 238 tumors (3.17%, fourth place).

Hu and Ch'in (*1*) in Peiping found 132 cases of cutaneous carcinoma in 821 carcinomas (16.2%, second rank). In Shanghai, Heine (*2*) reported 303 malignant tumors of which 103 were cutaneous (34%, first rank). Of 121 cancers reported by Dormanns (*3*) from Canton, 9 were skin cancers (7.43%, third place). From Hainan Island, Bercovitz (*4*) found 73 cases of skin cancer among 451 malignant tumors (16.18%, third place). In

TABLE 1.—Twenty most common malignant tumors in both sexes

Rank	Type	Number	Percent
1.	Carcinoma of cervix uteri	4, 448	36. 81
2.	Carcinoma of nasopharynx	1, 414	11. 70
3.	Carcinoma of breast	658	5. 45
4.	Carcinoma of stomach	636	5. 26
5.	Sarcoma, soft tissue	579	4. 79
6.	Carcinoma of large intestine	535	4. 44
7.	Carcinoma of mouth	403	3. 33
8.	Carcinoma of liver	390	3. 23
9.	Malignancies of lymphatic and hematopoietic tissues	390	3. 23
10.	Carcinoma of skin	282	2. 33
11.	Carcinoma of nasal cavity, middle ear, and accessory sinuses	264	2. 18
12.	Carcinoma of esophagus	245	2. 03
13.	Carcinoma of lung, trachea, and bronchus	212	1. 75
14.	Carcinoma of bladder and other urinary organs	176	1. 46
15.	Carcinoma of corpus uteri	169	1. 40
16.	Carcinoma of ovary	159	1. 32
17.	Carcinoma of larynx	151	1. 25
18.	Carcinoma of vagina	117	0. 97
19.	Carcinoma of penis	101	0. 84
20.	Carcinoma of kidney	87	0. 72
Total		12, 084	100. 00

TABLE 2.—Sixteen most common malignant tumors in males

Rank	Type	Number	Percent
1.	Carcinoma of nasopharynx	1, 046	22. 74
2.	Carcinoma of stomach	481	10. 46
3.	Sarcoma, soft tissue	351	7. 62
4.	Carcinoma of liver	347	7. 55
5.	Carcinoma of large intestine	325	7. 06
6.	Carcinoma of mouth	304	6. 61
7.	Malignancies of lymphatic and hematopoietic tissue	293	6. 37
8.	Carcinoma of esophagus	229	4. 98
9.	Carcinoma of nasal cavity, middle ear, and accessory sinuses	178	3. 87
10.	Carcinoma of lung, trachea, and bronchus	164	3. 57
11.	Carcinoma of skin	161	3. 50
12.	Carcinoma of larynx	136	2. 96
13.	Carcinoma of urinary bladder, ureter, and urethra	102	2. 22
14.	Carcinoma of penis	101	2. 20
15.	Carcinoma of testis	64	1. 39
16.	Carcinoma of kidney	50	1. 09
Total		4, 600	100. 00

1954 Yeh and Cowdry (*5*) in their paper, "Incidence of Malignant Tumors in Chinese, Especially in Formosa," based on 7,000 consecutive surgical specimens and 1,025 autopsies, found 84 skin cancers (69 carcinomas and 15 sarcomas) among 1,869 malignancies, 4.49 percent of all the tumors, fourth rank. The percentage or rank of skin cancer in Taiwan now is about the same as 10 years ago, though carcinomas of penis, vulva, and lip were excluded in the previous statistics but 4 cases of autopsy were added. It is obvious that the relative incidence of skin cancer in Taiwan is far lower than that reported from the mainland of China.

TABLE 3.—Sixteen most common malignant tumors in females

Rank	Type	Number	Percent
1.	Carcinoma of cervix uteri	4, 448	59. 43
2.	Carcinoma of breast	648	8. 66
3.	Carcinoma of nasopharynx	368	4. 92
4.	Sarcoma, soft tissue	228	3. 05
5.	Carcinoma of large intestine	210	2. 81
6.	Carcinoma of corpus uteri	169	2. 26
7.	Carcinoma of ovary	159	2. 12
8.	Carcinoma of stomach	155	2. 07
9.	Carcinoma of skin	121	1. 62
10.	Carcinoma of vagina	117	1. 56
11.	Carcinoma of mouth	99	1. 32
12.	Carcinoma of lymphatic and hematopoietic tissues	97	1. 30
13.	Carcinoma of vulva	87	1. 16
14.	Carcinoma of nasal cavity, middle ear, and accessory sinuses	86	1. 15
15.	Carcinoma of urinary bladder, ureter, and urethra	74	0. 99
16.	Choriocarcinoma	64	0. 86
Total		7, 484	100. 00

Apart from malignant melanomas and sarcomas the total number of carcinomas is 489. The numbers and types are: 385 epidermoid carcinomas, 58 basal-cell carcinomas, 35 intraepidermal carcinomas (Bowen's disease), 8 adenocarcinomas, and 3 carcinosarcomas. It is well known that most of the cutaneous carcinomas occur on the exposed surfaces where the majority are basal-cell carcinomas. The regional and sex distributions of the epidermoid carcinomas, basal-cell carcinomas, and intraepidermal carcinomas are shown in tables 4, 5, and 6, respectively. Of the 385 cases of epidermoid carcinomas only 23, or 5.97 percent, were on the trunk, whereas of 58 basal-cell carcinomas 11, or 18.97 percent, and of 35 intraepidermal carcinomas 15, or 42.86 percent, were on the trunk. This discrepancy is easy to explain because a majority of the tumors on the surfaces of the trunk were arsenical cancers, *i.e.*, 5 basal-cell carcinomas and 13 intraepidermal carcinomas. As will be discussed later, arsenical cancers are apt to develop on unexposed surfaces and multiplicity is one of their characteristics.

TABLE 4.—Epidermoid carcinoma (385 cases), by site and sex

	Male		Female		Total	
	Number	Percent	Number	Percent	Number	Percent
Head	41	18. 81	36	21. 55	77	20. 00
Lip	11	5. 04	4	2. 40	15	3. 90
Neck	2	0. 92	1	0. 60	3	0. 78
Trunk	14	6. 42	9	5. 39	23	5. 97
Penis	100	45. 87			100	25. 97
Vulva			80	47. 90	80	20. 78
Upper extremity	6	2. 75	9	5. 39	15	3. 90
Lower extremity	41	18. 81	28	16. 77	69	17. 92
Unknown	3	1. 38			3	0. 78
Total	218	100. 00	167	100. 00	385	100. 00

Adenocarcinoma is rarely seen in skin. Of the 8 cases in this series, 6 were in females of which 5 were located on the vulva; 2 were in males. Four of the 5 adenocarcinomas of the vulva probably originated in Bartholin's gland. Of the other 4 cases 2 were definitely of appendageal origin and the remaining 2 were included only because clinically no primary carcinoma was found.

TABLE 5.—Basal-cell carcinoma (58 cases), by site and sex

	Male		Female		Total	
	Number	Percent	Number	Percent	Number	Percent
Head	20	66. 67	22	78. 57	42	72. 42
Lip	2	6. 66	1	3. 57	3	5. 17
Trunk	8	26. 67	3	10. 72	11	18. 97
Upper extremity			1	3. 57	1	1. 72
Lower extremity			1	3. 57	1	1. 72
Total	30	100. 00	28	100. 00	58	100. 00

Carcinosarcoma is also a rare tumor of skin. However, there were 3 cases in this series and 1 of them was autopsied.

The distribution of all skin cancers by age is shown in table 7. Although the largest number of skin cancers falls in the age group 50–59, the age curve of ratios of skin cancer to all patients examined in each decade shows a steady increase up to over 80 (text-fig. 1).

TABLE 6.—Bowen's disease (35 cases), by site and sex

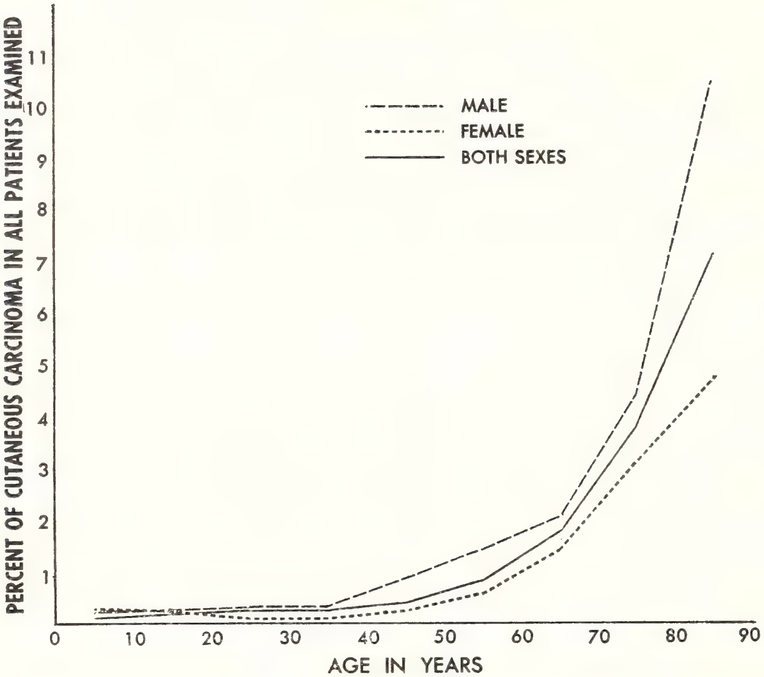
	Male		Female		Total	
	Number	Percent	Number	Percent	Number	Percent
Head	1	4. 17			1	2. 86
Lip	1	4. 17			1	2. 86
Trunk	12	50. 00	3	27. 27	15	42. 86
Upper extremity	1	4. 17	1	9. 09	2	5. 71
Lower extremity	9	37. 49	7	63. 64	16	45. 71
Total	24	100. 00	11	100. 00	35	100. 00

There were 52 cases of different sarcomas in this series, 9.23 percent of all skin cancers. Of the 52 sarcomas, 32 were in males and 20 in females: fibrosarcoma, 28; dermatofibrosarcoma, 3; liposarcoma, 7; angiosarcoma, 8 (lymphangiosarcoma, 3; hemangiopericytoma, 3; angiosarcoma, 2); malignant mesenchymoma, 2; and myxosarcoma, reticulum-cell sarcoma, Kaposi's sarcoma, and sarcoma unclassified, 1 each. Fibrosarcomas are included because they originate from subcutaneous connective tissue.

TABLE 7.—Age distribution of skin cancer*

Age	Lip			Carcinoma		Skin			Malignant melanoma			Total		
	Male	Female	Total	Penis male	Vulva female	Male	Female	Total	Male	Female	Total	Male	Female	Total
0–9							1	1					1	1
10–19														
20–29				4	13	6	6	12				10	19	29
30–39	1		1	16	17	14	17	31	5		5	36	34	70
40–49	5	1	6	39	18	38	21	59	3		3	85	40	125
50–59	6		6	27	22	48	32	80	4	2	6	85	56	141
60–69	2	1	3	11	12	37	26	63	3	3	6	53	42	95
70–79		3	3	4	5	15	16	31	2		2	21	24	45
80–89						3	2	5				3	2	5
Total	14	5	19	101	87	161	121	282	17	5	22	293	218	511

*Sarcomas excluded.



TEXT-FIGURE 1.—Ratio of cutaneous carcinoma to patients examined in each decade.

RELATIVE INCIDENCE OF SKIN CANCER BASED ON AUTOPSIES

Table 8 gives the number and percentage of autopsies by sex and age. This series includes 131 medicolegal cases of unknown sex and age, autopsied some 40 years ago, 702 stillbirths, and 462 neonatal deaths. There are a few malignant tumors in the former group and some congenital tumors and leukemia in the latter.

TABLE 8.—Number and percentage of autopsies, by sex and age

Age	Male	Female	Sex unknown	Total
Stillbirths	367	323	12	702
Neonatal deaths (under 1 month)	301	159	2	462
0–9	355	285		640
10–19	102	67		169
20–29	382	117		499
30–39	557	77		634
40–49	379	53		432
50–59	223	25		248
60–69	52	12		64
70–79	9	6		15
80–89	1	3		4
Age unknown	87	21	23	131
Total	2, 815	1, 148	37	4, 000

Of the 4,000 autopsies 570 (14.25%) were malignant tumors. The types and sites of the tumors, their numerical ranks, total number, and percentages are given in table 9. Forty-two types are represented by 1 or more samples according to the nomenclature and grouping adopted. Six were carcinoma of skin, 0.15 percent of all autopsies and 1.05 percent of all tumors, respectively, and rank fourteenth. There were 7 anatomically related tumors, 3 penile carcinomas, 3 malignant melanomas, and 1 vulvar carcinoma. The skin cancers constitute 0.33 percent of all autopsies and 2.28 percent of all malignant tumors, respectively, in eleventh place.

TABLE 9.—Cancers (570), according to site, numerical rank, number, and percentage

Rank	Type	Number of tumors	Percent of all autopsies	Percent of all tumors
1.	Carcinoma of liver	124	3. 10	21. 75
2.	Malignant disease of reticuloendothelial system	81	2. 03	14. 21
3.	Carcinoma of stomach	62	1. 55	10. 88
4.	Carcinoma of lung	48	1. 20	8. 42
5.	Intracranial tumors	39	0. 98	6. 84
6.	Carcinoma of esophagus	29	0. 73	5. 09
7.	Sarcoma of soft tissues	28	0. 70	4. 91
8.	Carcinoma of nasopharynx	27	0. 68	4. 74
9.	Carcinoma of uterus	18	0. 45	3. 16
10.	Carcinoma of large intestine	16	0. 45	2. 80
11.	Carcinoma of kidney	9	0. 23	1. 58
12.	Carcinoma of pancreas	9	0. 23	1. 58
13.	Malignant teratoma	7	0. 18	1. 23
14.	Carcinoma of skin	6	0. 15	1. 05
15.	Choriocarcinoma	6	0. 15	1. 05
16.	Carcinoma of oral cavity	6	0. 15	1. 05
17.	Carcinoma of ovary	5	0. 13	0. 87
18.	Retinoblastoma	5	0. 13	0. 87
19.	Carcinoma of breast	4	0. 10	0. 70
20.	Carcinoma of urinary bladder	4	0. 10	0. 70
21.	Carcinoma of penis	3	0. 08	0. 52
22.	Carcinoma of thyroid	3	0. 08	0. 52
23.	Carcinoma of testis	3	0. 08	0. 52
24.	Malignant melanoma	3	0. 08	0. 52
25.	Carcinoma of bile duct	2	0. 05	0. 35
26.	Carcinoma of pharynx	2	0. 05	0. 35
27.	Carcinoma of salivary gland	2	0. 05	0. 35
28.	Sarcoma of bone	2	0. 05	0. 35
29.	Carcinoma of thymus	1	0. 02	0. 18
30.	Carcinoma of gallbladder	1	0. 02	0. 18
31.	Carcinoma of ampulla of Vater	1	0. 02	0. 18
32.	Extragenital choriocarcinoma in male	1	0. 02	0. 18
33.	Carcinoma of prostate	1	0. 02	0. 18
34.	Carcinoma of larynx	1	0. 02	0. 18
35.	Carcinoma of appendix	1	0. 02	0. 18
36.	Carcinoma of middle ear	1	0. 02	0. 18
37.	Carcinoma of vulva	1	0. 02	0. 18
38.	Carcinoma of vagina	1	0. 02	0. 18
39.	Malignant mesothelioma	1	0. 02	0. 18
40.	Carcinoma of adrenal	1	0. 02	0. 18
41.	Carcinoma of urethra	1	0. 02	0. 18
42.	Primary undetermined	4	0. 10	0. 70
Total		570	14. 25	100. 00

Tables 10 and 11 give the site, rank, number, and percentage of malignant tumors in males and in females, respectively. In males, carcinoma of skin constitutes 0.18 percent of all autopsies and 1.08 percent of all tumors and ranks twelfth, whereas in females, it constitutes 0.09 percent of all autopsies and 0.97 percent of all tumors and ranks sixteenth. If 3 penile carcinomas and 2 malignant melanomas are added to the male group, the percentage will be 0.36 percent of all autopsies and 2.16 percent of all tumors, in tenth place. If 1 vulvar carcinoma is added to the female group, the percentage will be 0.17 percent of all autopsies and 1.94 percent of all tumors, in fourteenth place.

Table 10.—Cancers (464) in males, according to site, rank, number, and percentage

Rank	Site	Number of tumors	Percent of all tumors
1.	Carcinoma of liver	118	25. 43
2.	Malignant disease of reticuloendothelial system	67	14. 44
3.	Carcinoma of stomach	58	12. 50
4.	Carcinoma of lung	44	9. 49
5.	Intracranial tumors	29	6. 25
6.	Carcinoma of esophagus	25	5. 39
7.	Carcinoma of nasopharynx	23	4. 96
8.	Sarcoma of soft tissues	22	4. 74
9.	Carcinoma of large intestine	15	3. 24
10.	Carcinoma of kidney	8	1. 73
11.	Carcinoma of pancreas	6	1. 30
12.	Carcinoma of skin	5	1. 08
13.	Carcinoma of oral cavity	5	1. 08
14.	Malignant teratoma	4	0. 86
15.	Carcinoma of penis	3	0. 65
16.	Retinoblastoma	3	0. 65
17.	Carcinoma of urinary bladder	3	0. 65
18.	Carcinoma of testis	3	0. 65
19.	Malignant melanoma	2	0. 43
20.	Carcinoma of thyroid	2	0. 43
21.	Carcinoma of pharynx	2	0. 43
22.	Carcinoma of salivary gland	2	0. 43
23.	Sarcoma of bone	2	0. 43
24.	Carcinoma of bile duct	2	0. 43
25.	Carcinoma of gallbladder	1	0. 21
26.	Carcinoma of thymus	1	0. 21
27.	Carcinoma of ampulla of Vater	1	0. 21
28.	Extragenital choriocarcinoma	1	0. 21
29.	Carcinoma of prostate	1	0. 21
30.	Carcinoma of larynx	1	0. 21
31.	Carcinoma of appendix	1	0. 21
32.	Carcinoma of middle ear	1	0. 21
33.	Primary undetermined	1	0. 21
Total		464	100. 00

Heine (*2*) found no skin cancer in 106 autopsies, but Bonne (*6*) had 2 in 44 autopsied cancer cases in Chinese residents in Java. In this series 1.05 percent is much lower than any necropsy data from the United States (*7*).

TABLE 11.—Cancers (103) in females, according to site, rank, number, and percentage

Rank	Site	Number of tumors	Percent of all tumors
1.	Carcinoma of uterus	18	17. 48
2.	Malignant disease of reticuloendothelial system	14	13. 59
3.	Intracranial tumors	10	9. 71
4.	Choriocarcinoma	6	5. 83
5.	Sarcoma of soft tissues	6	5. 83
6.	Carcinoma of ovary	5	4. 86
7.	Carcinoma of liver	5	4. 86
8.	Carcinoma of stomach	4	3. 88
9.	Carcinoma of lung	4	3. 88
10.	Carcinoma of esophagus	4	3. 88
11.	Carcinoma of breast	4	3. 88
12.	Carcinoma of nasopharynx	4	3. 88
13.	Malignant teratoma	3	2. 92
14.	Carcinoma of pancreas	2	1. 94
15.	Retinoblastoma	2	1. 94
16.	Carcinoma of skin	1	0. 97
17.	Carcinoma of large intestine	1	0. 97
18.	Carcinoma of kidney	1	0. 97
19.	Carcinoma of urinary bladder	1	0. 97
20.	Carcinoma of thyroid	1	0. 97
21.	Carcinoma of vulva	1	0. 97
22.	Carcinoma of vagina	1	0. 97
23.	Malignant mesothelioma	1	0. 97
24.	Carcinoma of adrenal	1	0. 97
25.	Carcinoma of urethra	1	0. 97
26.	Carcinoma of oral cavity	1	0. 97
27.	Primary undetermined	1	0. 97
Total		103	100. 00

CARCINOMA OF PENIS

The extensive literature existing on the geographical and racial differences of carcinoma of the penis is not reviewed here. High frequencies of penile carcinoma relative to other tumors have occurred in some parts of China. Almost all the authors, who wrote on tumors in the Chinese, state that penile cancer is more common in Chinese than in Europeans. The percentages reported from various parts of China are: Tsinan, 19.8 percent (*8*); Canton, 6.6 percent (*3*); Shanghai, 8.2 percent (*2*); Hainan Island, 12.6 percent (*4*); Peiping, 11.4 percent of all carcinomas and 8.9 percent of all malignant tumors (*1*); Chinese immigrants in Indonesia, 15.1 percent (*9*). However, in our surgical series, there were 101 cases among 12,084 tumors, only 0.84 percent of all tumors and 2.2 percent of all cancers in males, and in the 4,000 autopsies there were only 3 cases, 0.08 percent of all autopsies, 0.52 percent of all cancers, or 0.65 percent of all cancers in males. Other than 1 intraepidermal carcinoma, the 100 are exclusively epidermoid carcinoma. Histological grading of the 100 tumors revealed: Grade I, 40; Grade II, 33; Grade III, 15; Grade IV, 4; and grades of 8 cases were undetermined because of an inadequate specimen. The age distribution shows 4 in age group 20–29, 16 in 30–39, 39 in 40–49, 27 in 50–59, 11 in 60–69, and 4 in 70–79. A majority occurred above 40.

Lymphadenectomy of inguinal lymph nodes was done in 20 patients and 7 showed metastatic carcinoma.

Since penile carcinoma is a visible, disfiguring, and unpleasant tumor surely not to be neglected by the patient himself, then, to what can this large difference be attributed? Most authors stated that the high frequency of penile carcinoma is the result of phimosis and lack of bodily cleanliness. Reasonable as this may seem, Hu and Ch'in (*1*) would not accept this explanation. Based on the incidence of penile papilloma in an age group 15 years younger than that of penile carcinoma, they suggested that many penile carcinomas probably began as benign papillomas. But it is our experience that penile papilloma is less common than penile carcinoma in Taiwan. Owing to warmer weather, better living conditions, more general education, etc., bodily uncleanliness may be less important in Taiwan. Are those improvements alone responsible for the difference in the frequency of penile carcinoma? As 34 of the 101 patients with penile carcinoma had complete phimosis, it is conceivable that phimosis plus bad personal hygiene may be the etiological factor in inducing penile carcinoma.

MALIGNANT MELANOMA

The frequency of malignant melanoma in the Chinese is confusing. In Hu and Ch'in's report (*1*) from Peiping, they had 13 malignant melanomas among 1,050 malignant tumors. However, Dormanns (*3*) found 6 cases among 121 cancers in Canton. In our surgical series there were 22 cases definitely originating from skin, 0.18 percent of all malignancies. The sex distribution shows 17 in males and 5 in females. The ages range from 25 to 72, with 14 tumors occurring in individuals over 50. On the lower extremity there were 63.63 percent (heel 4, sole 4, toe 3, dorsum of foot 1, thigh 1, and lower leg 1), head 18.18, scrotum 4.54, perineum 4.54, inguinal region 4.54, and finger 4.54 percent.

In addition to the 22 cases originating from skin, there were 8 from eye, 4 from nasal mucosa, 2 from oral mucosa, and 8 of unknown origin. Since in 4 of the last 8 the first biopsy was obtained from the peripheral lymph node, the primary site was probably the skin. The 44 cases constitute a percentage of 0.36 which is still low.

Of the 4,000 autopsies, there were only 3 malignant melanomas, revealing the same percentages as for penile carcinoma, *i.e.*, 0.08 percent of all autopsies and 0.52 percent of all tumors. Compared with data from the United States, it is apparently lower than any of them (*10*).

ARSENICAL CANCER

As mentioned previously, 52 cases of arsenical cancer are included in this series. All these patients are from a limited area on the southwest coast of Taiwan, with a population of approximately 160,000. In this area artesian wells as a source of drinking water have been used for 40 years. A majority of the artesian wells have a relatively high concentration of arsenic, ranging from 0.8 to 2.5 ppm. Among the inhabitants of this area a remarkable percentage of chronic arsenical intoxication, manifested as hyperpigmentation, keratosis, and cancer, was discovered in 1960. Cases of arsenical cancer due to high concentration of arsenic in the public water supply have been described as occurring in Reichenstein (Silesia) and Cordoba in Argentina (*11*). It was stated that the dietary arsenical cancer presented all the major characteristics of medicinal arsenical cancer which occurs after prolonged arsenic medication (Fowler's solution, etc.) for certain skin diseases, especially psoriasis (*11–13*). In medicinal arsenical cancer, patients with cancers developing in mucous membranes (*14*) and internal organs (*12*) have been reported. To date, we have found 64 cancers in 52 patients in this area: 32 males and 20 females. Of these 64 cancers, 17 are epidermoid carcinoma, 8 basal-cell carcinoma, and 39 intraepidermal carcinoma or Bowen's disease. We have not yet seen a single cancer originating from either mucous membrane or internal organ.

In patients with arsenical cancer, the parts particularly affected are fingers, legs, feet, toes, and trunk. The site distribution of the 64 cancers in this series is shown in table 12. Thirty (46.84 percent) are located in unexposed parts of the body. The number of cancers on hands, fingers, feet, and toes does not represent the actual incidence because removal of small lesions on such places for histological examination has to be avoided, otherwise it would keep those patients from daily work.

The distribution of all arsenical cancers in each sex by age (table 13) shows that 73.07 percent occurs at ages over 50 and that in females cancer developed 1 decade earlier than in males.

TABLE 12.—Site distribution of 64 arsenical cancers

Site	Epidermoid carcinoma		Basal-cell carcinoma		Bowen's disease		Total	
	Male	Female	Male	Female	Male	Female	Male	Female
Scalp			1				1	
Finger	1	1					1	1
Hand				1	2	1	2	2
Arm					2	1	2	1
Shoulder	1				1		2	
Chest	2		1				3	
Abdomen	1	1		2	7		8	3
Back			2	1	5	5	7	6
Vulva		1						1
Thigh	1	1			3	3	4	4
Knee						1		1
Popliteal fossa					1	1	1	1
Lower leg	4	1			4	1	8	2
Foot	2						2	
Toe						1		1
Total	12	5	4	4	25	14	41	23

TABLE 13.—Distribution of arsenical cancer, by sex and age

Sex	Age						Total
	20–29	30–39	40–49	50–59	60–69	70–79	
Male		3	4	10	10	5	32
Female	2		5	9	3	1	20
Total	2	3	9	19	13	6	52

Multiplicity of arsenical cancer is found in over 50 percent of the cases (*15*). According to Sommers *et al.* (*12*), in 143 published cases with medicinal arsenical epitheliomas, 71.3 percent were multiple. In this series, multiple cancers were found in 12 patients. The combinations were as follows:

Epidermoid carcinoma + epidermoid carcinoma	1
Epidermoid carcinoma + Bowen's disease	5
Basal-cell carcinoma + basal-cell carcinoma	1
Basal-cell carcinoma + Bowen's disease	2
Bowen's disease + Bowen's disease	3

The percentage of multiple cancers in this series (23.05%), which is far lower than that reported in the literature, does not represent the actual figure, simply because it is impossible in a field survey to remove all the lesions from 1 patient at the same time. Among the 52 patients there is a woman who has at least 9 tumors which can be diagnosed macroscopically as skin cancers, but only 1 biopsy was taken for histological diagnosis. In chronic arsenism, hyperpigmentation, keratosis, and cancer are the main characteristic changes of the skin. There may also be leukoderma, warts, and papillomas, and invisible transitions are frequently encountered.

Hyperpigmentation occurs everywhere over the body and in fully developed cases usually appears as diffuse dappling of dark brown, particularly marked in unexposed parts of the body such as the trunk, buttock, and upper part of thigh (fig. 1). Spots of depigmentation, not exceeding 2 or 3 mm in diameter, are frequently encountered. Microscopically, the principal pigmentary change is the hyperpigmentation of the basal and adjacent prickle cells and melanocytes. This is especially marked in atrophic epidermis, while in hyperplastic epidermis accumulation of melanophages seems to be the main source of pigmentation. Slight lymphocytic infiltration around vessels may occasionally be present. Basophilic degeneration of collagenous fibers is marked in severe cases.

Grossly, arsenical keratosis is characterized by a sessile, conical, or wartlike projection of often pigmented keratin (fig. 2). Microscopically it appears as a thick, dense, usually pinkish, sometimes irregularly stained horny layer. The granular layer is usually well developed, but may be thin or absent in some lesions. Parakeratosis is not always present. The prickle-cell layer is more or less widened and most of the cells are acidophilic and PAS-positive. Prolongation of rete ridges may occur. Lymphocytic infiltration in the upper layers of the dermis may or may not be present. Diminution or loss of elastic fibers in the dermis is sometimes pronounced. Cornlike arsenical keratosis (fig. 3) shows almost the same picture as that of verruca vulgaris except for lack of inclusion bodies (fig. 4).

Some keratotic lesions show cellular atypia including irregular size of cells, basophilic cytoplasm, irregular increase in nuclear size, multinucleated giant cells, increased number of mitotic figures, typical or atypical mitosis, and focal individual cell keratinization, all of which are like those of Bowen's disease (fig. 5). In such lesions vacuolar changes in the cytoplasm or nucleus are usually, though not always, present (fig. 6), but not pathognomonic in arsenical keratosis or cancer.

Invasive arsenical carcinoma occurs not only in exposed but also on a considerable proportion in unexposed parts of the body. Epidermoid carcinoma is more common than basal-cell carcinoma. Six of 8 basal-cell carcinomas were located on the trunk (figs. 7 and 8). The epidermoid and basal-cell carcinomas vary from small to quite large ulcers, up to 15 cm in diameter in advanced cases. This is sometimes due to spontaneous ulceration, but often the infection is caused by the patient himself who peels or cuts the horny layers of the lesion with contaminated nails or knives. Two patients with epidermoid carcinoma showed metastasis and 1 died of carcinoma. The grade of differentiation of component cells of the epidermoid carcinoma varied from Grade I to Grade IV (figs. 9 and 10) and the percentage of each grade was about the same as for ordinary epidermoid carcinoma of the skin. The pattern of growth of the basal-cell carcinoma was also variable: adenoid cystic, trichoepitheliomatous, hyperpigmented, etc., as in ordinary basal-cell carcinoma.

To know the incidence of chronic arsenical intoxication in this area, a general survey, particularly on signs of chronic arsenism, was made recently in 6 villages. Details of the composition of the population of these 6 villages together with that of the persons examined are given in table 14. The number and percentage of persons of each sex in each decade of life are recorded. Of the whole population, 80.7 percent was examined and the percentage examined in each decade is almost the same as for the whole population. The number and percentage of persons who show any or all of the 3 main changes are shown in table 15. Although the incidence of chronic arsenism averages 36.8 percent, it is apparent that the percentage increases markedly with age and that 84.3 percent over 50 shows typical skin changes. Generally speaking, the incidence in females is somewhat lower than that in males.

In this survey, 35 skin cancers were found, an incidence of 1.11 percent, which is about 25 to 30 times that in western countries (table 16). The percentage of skin cancer also markedly increases with age. It is 6.8 percent in persons over 50.

In addition to the high frequency of arsenical carcinoma in this area, there is an endemic peripheral vascular disorder resulting in gangrene of extremities, especially the feet, named Blackfoot disease by the local people because it is black (*16–18*). Pathologic examination of 3 autopsies and 32 amputated legs, 2 feet, and 1 finger in 33 Blackfoot patients disclosed 5 associated with skin cancer, of which 2 showed multiple

Table 14.—Composition of the population of 6 villages surveyed—number and percent examined, by sex and age

Sex	Population	Age									Total	Grand total
		0–9	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89		
Male	Number	647	447	249	223	196	133	91	29	3	2, 017	
	Percent	16. 43	11. 35	6. 32	5. 66	4. 98	3. 38	2. 31	0. 74	0. 08	51. 22	
Female	Number	558	428	295	240	169	126	64	24	5	1, 921	3, 938
	Percent	14. 17	10. 87	7. 49	6. 09	4. 29	3. 20	1. 63	0. 60	0. 12	48. 78	100. 0
Male	Number	600	331	158	164	156	114	88	24	2	1, 637	
	Percent	18. 88	10. 42	4. 97	5. 16	4. 91	3. 59	2. 77	0. 76	0. 06	51. 51	
Female	Number	484	260	196	221	174	102	56	22	2	1, 541	3, 178
	Percent	15. 23	8. 18	6. 17	6. 95	5. 48	3. 21	1. 76	0. 70	0. 06	48. 49	100. 0

Table 15.—Number and percent of patients with signs of chronic arsenical intoxication, by sex and age

		Age									Total	Grand total
		0–9	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89		
Number examined	Male	600	331	158	164	156	114	88	24	2	1, 637	
	Female	484	260	196	221	174	102	56	22	2	1, 541	3, 178
Male	Number	17	88	86	109	108	106	81	24	2	601	
	Percent	2. 8	20. 5	54. 4	66. 5	69. 2	93. 0	92. 0	100. 0	100. 0	36. 5	
Female	Number	12	50	85	140	142	77	41	21	2	570	1, 171
	Percent	2. 5	19. 2	43. 4	63. 3	81. 6	75. 5	73. 2	95. 5	100. 0	37. 0	36. 8

cancers (*19*)—obviously more than a coincidence. Knowing that most of the deep artesian wells show a variably high concentration of arsenic, that the patients subject to Blackfoot disease are limited to people using deep well water for drinking purposes (*20*), and that there is a tendency to develop arsenical cancer in Blackfoot patients, we thought of the intimate relationship between Blackfoot disease and chronic arsenical intoxication. Thus, we re-examined the 225 patients with Blackfoot disease for the signs of chronic arsenical intoxication. The results are shown in table 17. It is not surprising that 81.8 percent shows skin lesions of chronic arsenical intoxication, because here the younger age group, below 20 years, made up only 3.1 percent of the total while there are more than 50 percent in this age group in the general population. However, percentages in the second, third, and fourth decades, respectively, are much higher in Blackfoot patients than in the general population. Among these 225 patients, 18 have developed arsenical carcinoma up to the present time, an incidence of 8 percent, more than 7 times that in the general population. Of the 18 patients with cancer, 14 are over 50, an incidence of 13.1 percent, about 2 times that in the general population. Therefore, it would seem justifiable to assume that the Blackfoot patients are apt to develop signs of chronic arsenical intoxication or arsenical carcinoma, or that people heavily intoxicated with arsenic tend to develop Blackfoot disease or arsenical carcinoma.

Clinically, the symptoms and course of Blackfoot disease are more similar to the clinical picture of thromboangiitis obliterans than that of any other peripheral vascular disease (*16*). Pathologically, about one fourth of the patients have vascular changes as seen in Buerger's disease, while the remaining three fourths show changes of arteriosclerosis obliterans. As the cause of both Buerger's disease and arteriosclerosis obliterans is still unknown, it is beyond the scope of this paper to discuss these diseases in detail. Despite the poor diet, especially fat deficiency, the preponderance of marked arteriosclerosis obliterans in Blackfoot patients among the inhabitants in this area is, however, of significance in the investigation of the etiology of the disease.

The exposure time for development of arsenical hyperpigmentation, keratosis, and cancer varies greatly from village to village and from individual to individual in the same village. The earliest appearance of hyperpigmentation was found in school children of 7 or 8 years, and the earliest cancer was seen in a woman of 24. The arsenical keratosis occurs usually a few years later than hyperpigmentation.

It is interesting to discuss the findings from a group of saltern workers who had lived in the same area for variable years and moved at different ages to another saltern in Kaohsiung county, some 50 miles south of the affected area, where the drinking water from the shallow wells is not contaminated by arsenic. The number of persons in this group is 94: 48 males and 46 females. The age distribution is shown in table 18, and the number of patients with arsenical skin lesions by age and years of exposure is given in table 19.

TABLE 16.—Distribution of 35 patients with arsenical carcinomas, by sex and age

	Age																			
	1-9		10-19		20-29		30-39		40-49		50-59		60-69		70-79		80-89		Total	
Sex	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Total
Examined	600	484	331	260	158	196	164	221	156	174	114	102	88	56	24	22	2	2	1,637	1,541	3,178
Number with arsenical carcinomas						1	2		1	3	8	9	3	3	4	1			18	17	35
Percent with carcinomas						0.51	1.21		0.64	1.72	7.02	8.82	3.41	5.36	16.66	4.54			1.10	1.12	1.11

Table 17.—Number and percent of 225 patients with Blackfoot disease with signs of chronic arsenical intoxication, by sex and age

		Age								
		10–19	20–29	30–39	40–49	50–59	60–69	70–79	Total	Grand total
Number with Blackfoot disease	Male	3	12	18	29	44	25	5	136	
	Female	5	15	19	17	22	10	1	89	225
Patients with skin lesions:										
Male	Number		11	16	24	40	24	5	120	
	Percent		91. 7	84. 2	82. 8	90. 9	96. 0	100. 0	88. 2	
Female	Number	3	10	14	14	15	7	1	64	184
	Percent	60. 0	66. 6	73. 3	82. 4	68. 2	70. 0	100. 0	71. 9	81. 8

TABLE 18.—Age distribution of 94 saltern workers

Age	20–29		30–39		40–49		50–59		60–69		70–79		80–89		Total		Grand total
Sex	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	
Number	2	4	5	6	11	14	13	12	13	6	4	3		1	48	46	94

TABLE 19.—Number and percent of patients with arsenical skin lesions, by age and years of exposure

Years of exposure	1–4	5–9				10–14				15–19					20–24				
Age	40–49	20–29	30–39	40–49	50–59	20–29	30–39	40–49	50–59	20–29	30–39	40–49	50–59	60–69	20–29	30–39	40–49	50–59	60–69
Number of persons examined	1	3	1	2	2	1	1	3	2	1	5	11	6	4	1	4	4	9	6
Number of patients with signs of CAI*					2			3	2	1	3	8	5	4		3	3	6	6
Hyperpigmentation					2			3	2	1	3	7	5	3		3	2	6	3
Keratosis												1	1	1			1	1	3
Cancer																		1	
Number of patients with BFD*																			

Years of exposure	25–29				30–34			35–39		40–44	45–49	Total	
Age	40–49	50–59	60–69	70–79	50–59	60–69	70–79	40–49	50–59	80–89	50–59	Number	Percent
Number of persons examined	2	1	8	2	1	2	5	1	3	1	1	94	100.0
Number of patients with signs of CAI*	2	1	6	1	1	2	5	1	3	1	1	70	74.5
Hyperpigmentation	2	1	5	1	1	2	4	1	2	1	1	61	64.89
Keratosis		1	2	1			3	1	3	1		20	21.27
Cancer	1		1	1			2					6	6.38
Number of patients with BFD*								1	1			3	3.19

*CAI: chronic arsenical intoxication; BFD: Blackfoot disease.

It is obvious that hyperpigmentation can be seen only in patients who had been exposed for at least 5 years, keratosis for 15 years, and cancer for 20 years. On the other hand, keratosis or cancer does not necessarily occur even after an exposure of 45 years and even 25.5 percent show no signs of chronic arsenical intoxications. A considerable number of persons once exposed to arsenic intoxication for a certain time develop skin lesions of chronic arsenism sooner or later, even after no further contact with contaminated water.

The latent period for arsenical cancer is long; in some patients, cancer occurred even 30 to 40 years after discontinued use of contaminated water. This is a serious problem to the younger generation living in the affected area who may develop arsenical cancer many years after contaminated water has not been used for drinking purpose.

ETIOLOGICAL FACTORS

The etiological factors generally accepted for human skin cancer vary greatly. Based on a study of the clinical data available in this series, 149 cases are associated with or preceded by various chronic diseases or pathological conditions which may be contributory to the development of skin cancer. Table 20 shows the relationship between the various chronic diseases or pathological conditions and types of skin cancer.

TABLE 20.—Relationship between various pathological conditions and skin cancer

	Types of skin cancer									
	Epidermoid carcinoma		Basal-cell carcinoma		Bowen's disease		Malignant melanoma		Total	
Pathological conditions	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Chronic arsenical intoxication	11	5	4	3	16	13			31	21
Phimosis	34								34	
Trauma	6	2					4	2	10	4
Chronic ulcer	6	3	2						8	3
Chronic osteomyelitis	7	1							7	1
Scar										
Burn	2	3							2	3
Operation	2								2	
Moxibustion	1								1	
Chronic infection	1	2	2						3	2
Kraurosis		5								5
Ionizing radiation	2	1							2	1
Leukoplakia		3								3
Chronic otitis media	2								2	
Cautery							1		1	
Electric cauterization				1						1
Alkali solution				1						1
Hyperkeratosis	1								1	
Total	75	25	8	5	16	13	5	2	104	45

Generally speaking, men developed more cancers than women, even in the same environment. This sex difference may be easily explained in some cancers but not in others. In cancers originating from pre-existing chronic lesions, including chronic ulcers, burn scars, trauma, chronic dermatitis, etc., the higher frequency in males seems simply due to the fact that the risk of developing such chronic lesions is greater in men because of more outdoor activities. However, in chronic arsenical intoxication from drinking water containing a high concentration of arsenic, the exposure is the same in both sexes, yet cancer is more prevalent in males. Could it be explained by the assumption that men consume more water than women?

It is generally agreed that chronic ulcers, whether tropical or secondary to osteomyelitis and other chronic infections, are important in the development of skin cancer, especially on exposed surfaces. Since the introduction of antibiotics, chronic leg ulcer is a rarity in Taiwan. All the chronic ulcers listed in table 20 should be considered as results of inadequate treatment or negligence by the patients themselves. Some of them had had ulcers for 20 years, and not a few of them had been treated by herb doctors with an herb drug of unknown nature.

Trauma as a cause is still controversial. However, among 22 malignant melanomas, 6 patients had trauma of various kinds directly on a nevus, which gave rise to a malignant melanoma after a period of 3 months to 2 years.

As basal-cell carcinoma is far less common than epidermoid carcinoma in this series, and solar keratosis, a precancerous lesion of skin, and other relating lesions are also rare in Taiwan, sunlight does not seem to be as important for the induction of skin cancer in Chinese as in Caucasians (*10*)

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PLATES

PLATE 5

FIGURE 1.—Typical arsenical hyperpigmentation of skin in a 34-year-old male. *Note* diffuse dappling of dark brown.

FIGURE 2.—Arsenical hyperpigmentation and keratosis in a 53-year-old male. Disseminated dark patches, actually slightly elevated, are keratotic lesions, some of which may have converted to intraepidermal carcinoma.

FIGURE 3.—Arsenical keratosis in a 62-year-old male. *Note* numerous verrucous lesions on dorsum of hands and forearms.

PLATE 6

FIGURE 4.—Histologic section of verrucous lesion of arsenical keratosis on right arm of a 68-year-old male. *Note* hyperkeratosis, acanthosis, and some elongation of rete ridges.

FIGURE 5.—Histologic section of an intraepidermal carcinoma on right knee of a 53-year-old female, showing irregular size of cells, atypical mitotic figures, multinucleated giant cells, and individual cell keratinization.

FIGURE 6.—Histologic section of an intraepidermal carcinoma on back of a 73-year-old male with marked vacuolar changes in cytoplasm and nucleus.

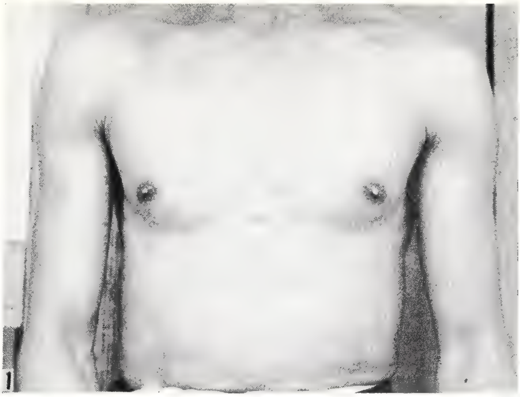
PLATE 7

FIGURE 7.—Histologic section of a basal-cell carcinoma on left flank of a 67-year-old female, showing slender ribbons of basal cells.

FIGURE 8.—Histologic section of another basal-cell carcinoma on back of same patient, showing some adenoid cystic structure.

FIGURE 9.—Histologic section of an epidermoid carcinoma, Grade 1, on right sole of a 57-year-old male.

FIGURE 10.—Histologic section of an epidermoid carcinoma, Grade 4, on left vulva of a 28-year-old female with metastasis in left inguinal lymph nodes.



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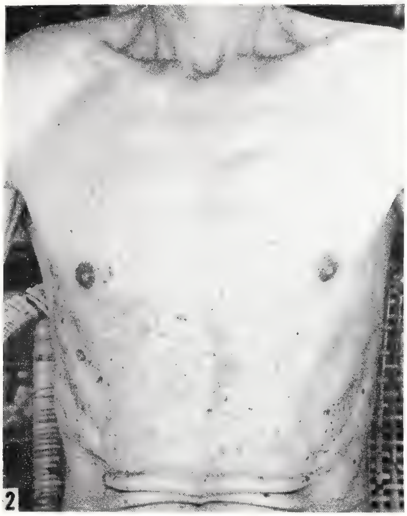

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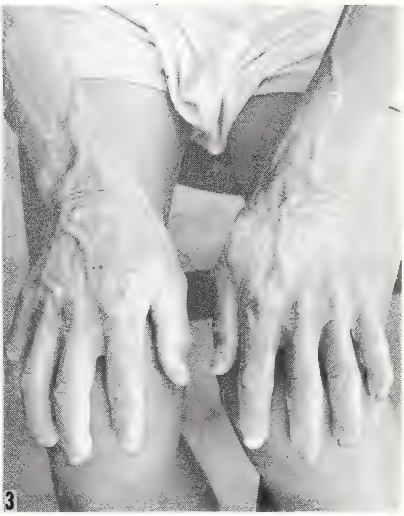

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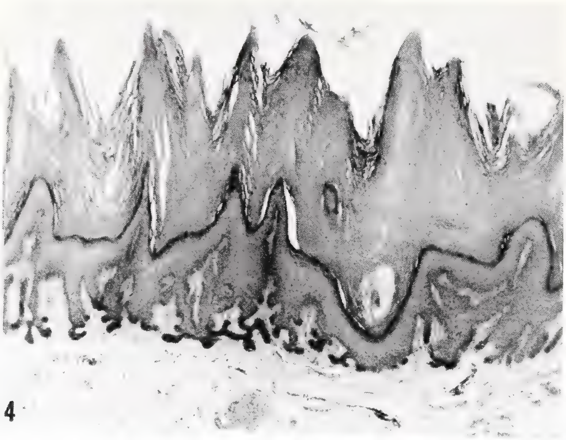
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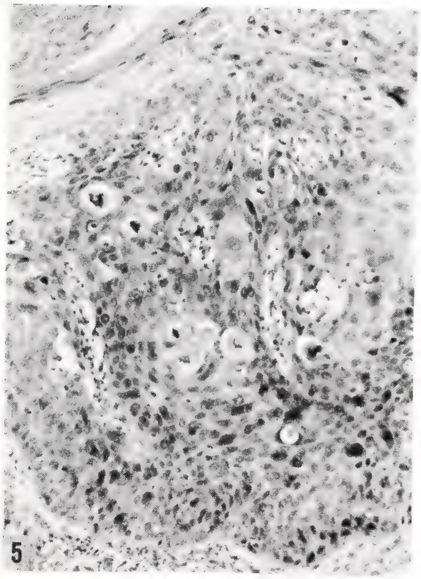
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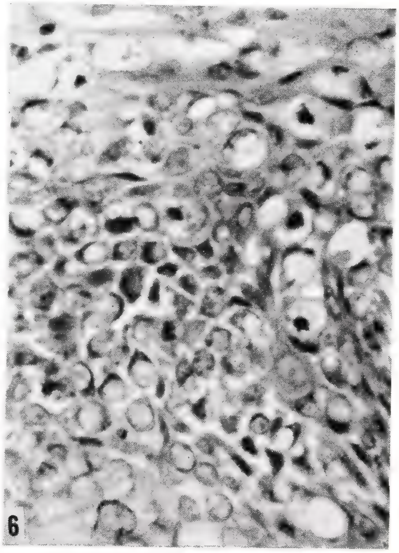
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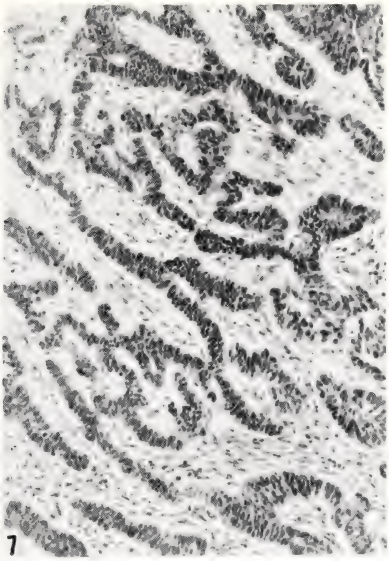

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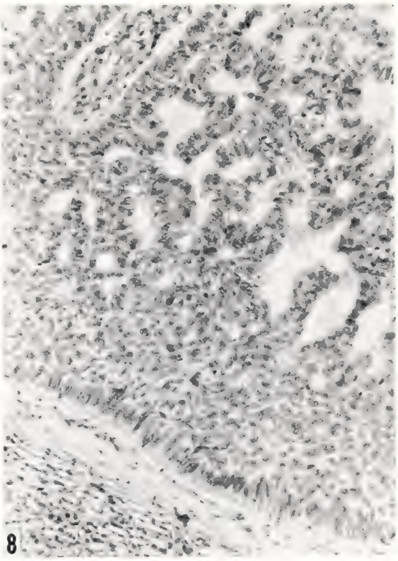

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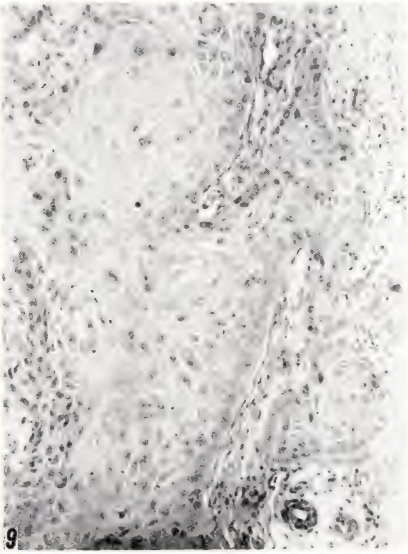

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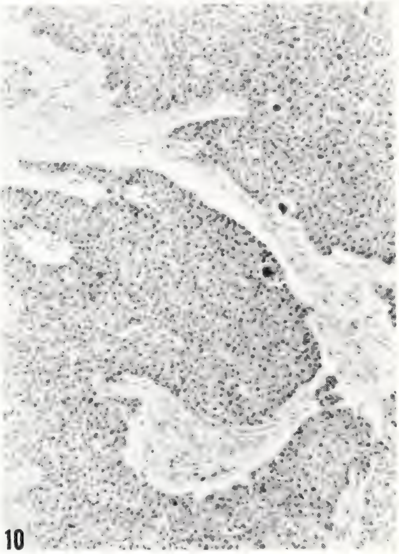

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OBSERVATIONS ON THE INCIDENCE, BIOLOGY, AND PATHOLOGY OF SKIN CANCER AMONG FILIPINOS[1]

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SUMMARY

Although infectious diseases remain the major cause of death among Filipinos, cancer is gradually becoming a public health problem. In 1960, it ranked seventh among the 10 major causes of death in the Philippines. The 10 most common sites of cancer in the 3 largest general hospitals in Manila were: the uterus, breast, oral cavity, skin, lymph nodes, colon, stomach, liver, nasopharynx, and ovary. This study describes the incidence and biology of skin cancer in several hospitals and institutions in the Philippines. For every 100,000 patients admitted in a general hospital, 10 to 15 are afflicted with skin cancer. The clinical records of 330 patients with cancer of the skin were reviewed; 24.5 percent were from 40 to 60 years of age. More men suffered from this neoplasm than women, although 61.5 percent of the latter had basal carcinomas. The head and neck areas and other exposed regions of the body were frequent sites in 67 percent of reported cases. The pathological features described disclosed the following histologic types: epidermoid carcinomas, basal-cell carcinomas, melanomas, adnexal carcinomas, and other rare malignant neoplasms of the skin. From the data submitted, the etiopathogenesis of these tumors was traced. Case reports were discussed and their potential causes evaluated, such as, solar radiation, trauma, burns, ultraviolet rays, ionizing radiation, chemicals, infections, occupation, diet, and heredity. The incidence of skin cancer among Chinese, American, and European residents in the Philippines revealed interesting statistical differences, and pertinent socioeconomic factors were considered.—NCI Monogr. No. 10: 109–125, 1963.

TO GATHER true and accurate information on the incidence of cancer in the Philippines is a difficult task. Our country is composed of 7,083 islands inhabited by 55 ethnic segments with 90 languages and 123 dialects. The lack of adequate government support, plus social and cultural differences, presents problems for the cancer researcher.

However, available significant data have shown that cancer has become a public health problem in the Philippines, though infectious diseases remain the major cause of death. The mortality trends for the past

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

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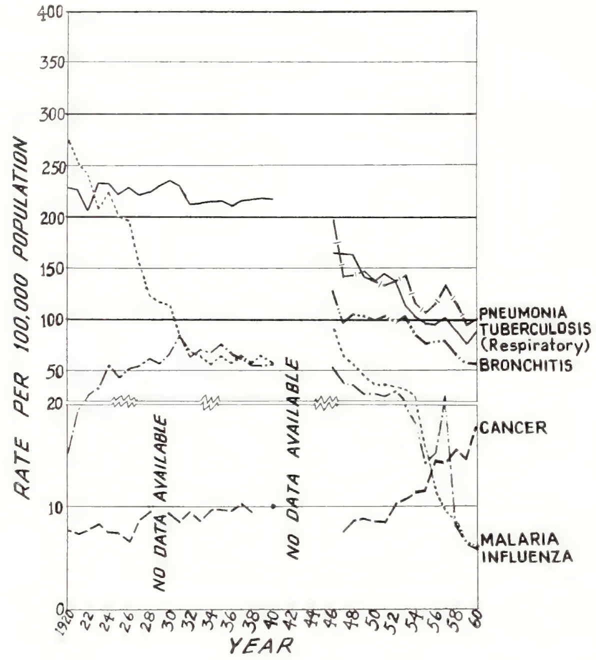
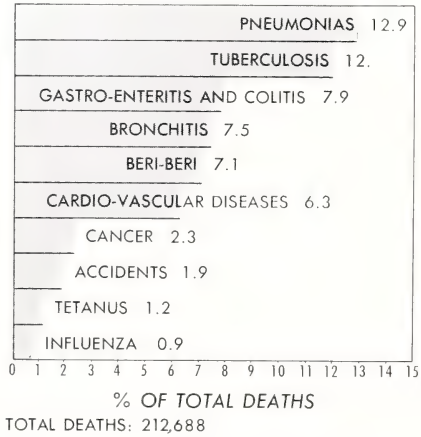
40 years (1920–60) of selected notifiable diseases and leading causes of death in the Philippines disclosed that tuberculosis, pneumonia, influenza, and malaria are declining (text-fig. 1). This has been brought about by effective therapeutic measures, improvement in environmental conditions, public awareness of the hazards of communicable diseases, and



TEXT-FIGURE 1.—Mortality trends of selected notifiable diseases compared to cancer; mortality rate per 100,000 population (1920–60).



TEXT-FIGURE 2.—Leading causes of death; percentage distribution (1960).

knowledge of modern control of infectious diseases through systematic health educational campaigns. Cancer was not among the leading causes of death in 1951, but in 1960 it ranked seventh (text-fig. 2). Of the 212,688 registered deaths in 1960, 2.3 percent were due to cancer (*1*). This increase could be a reflection of better diagnosis, higher degree of cancer consciousness, and the steady increase in the lifespan of the Filipino population.

Our studies of the mortality and morbidity from cancer in 3 general hospitals in Manila and in records of the Philippine Cancer Society showed that the 10 most common sites of cancer were the uterus; breast; oral cavity; skin; reticuloendothelial lymph nodes; colon and anal canal; stomach; liver; nasopharynx; and ovary (table 1).

This paper is a review and study of the incidence and biology of skin cancer reported in these hospitals and institutions.

MATERIAL

The main source of data for study was from the files of three institutions: the University of Santo Tomas Hospital, North General Hospital, and the Philippine Cancer Society, in the Manila area. These institutions cater to a city population, at risk estimated to be 1,399,391, consisting of permanent residents, suburbanites, and provincial visitors. These hospitals were chosen because of their large number of admissions, complete histopathological data, and availability of clinical records. Their outpatient departments examine approximately 150,000 to 200,000 patients a year. The patients also are subjected to the same altitude, temperature, humidity, nutrition, and local customs. For additional data, questionnaires were sent to suburban and distant hospitals, clinics, and medical societies throughout the Philippine Islands. The medical directors of mining companies, tanneries, chemical factories, and oil and petroleum firms were asked to contribute information regarding skin cancer. Responses from these sources were screened. Other data were excluded for lack of clinical workup and histopathological examination. The bulk of the patients suffering from skin cancer came from Manila and nearby provinces.

HOSPITAL INCIDENCE

An analysis of the comparative incidence of the 10 most common cancers was made from complete records obtained from the 3 largest general hospitals in Manila. In the University of Santo Tomas Hospital, 1,225 malignant tumors were found from 1953–59. Of this material, 109 were skin cancers (10.8%), which ranked skin cancer fourth in incidence. This hospital has a total yearly admission of 107,000 patients (table 1). From the North General Hospital, 1946–60, clinical records showed that

146 were skin cancers of 2,405 malignant neoplasms. This represents 6.0 percent of all cancers in that hospital and places skin cancer fourth in rank. The North General Hospital has an average annual admission of 200,000 patients. In a 10-year survey from 1947–56, the Philippine General Hospital statistics revealed that 236 skin cancers were observed, which represented 5.4 percent of a total of 4,368 malignancies. An average of 250,000 patients a year was examined and admitted. This hospital is the biggest in the Philippines and its material for study is the largest ever assembled for analysis (*2*).

TABLE 1.—Comparative incidence of the 10 most common cancers at different sites in 3 general hospitals in Manila*

	USTH (1953–59)			NGH (1946–60)			PGH (1947–56)		
Site	Rank	Number	Percent	Rank	Number	Percent	Rank	Number	Percent
Uterus	1	165	13. 47	1	787	32. 2	3	317	7. 30
Breast	2	152	12. 4	2	264	10. 5	2	515	11. 80
Oral muscosa, palate, and tongue	3	133	10. 86	7	100	4. 1	1	717	16. 41
Skin	4	109	8. 9	4	146	6. 0	4	236	5. 40
Reticuloendothelial lymph nodes	5	94	7. 67	8	92	3. 4	7	98	2. 25
Colon and anal canal	6	82	6. 6	6	101	4. 19	5	212	4. 85
Stomach	7	64	5. 22	13	26	1. 08	6	112	2. 56
Liver	8	42	3. 43	14	22	0. 91	9	61	1. 37
Nasopharynx	9	41	3. 35	12	28	1. 12	10	28	0. 64
Ovary	10	34	2. 84	5	120	4. 98	8	78	1. 80
Total malignancy		1, 225			2, 405			4, 368	

*USTH = University of Santo Tomas Hospital; NGH = North General Hospital; PGH = Philippine General Hospital.

Amazingly, even in this big hospital, skin cancer ranked fourth in occurrence of all the total malignancies.

From these figures, one can observe that skin cancer ranks fourth in all three general hospitals and this can be taken as a baseline for hospital incidence of malignancies of the skin. Statistically, it means that for every 100,000 patients admitted 10 to 15 are suffering from cancer of the skin. The mortality statistics from these institutions do not reflect the true frequency of these skin tumors because of their high rate of curability.

AGE AND SEX INCIDENCE

The clinical records of 330 cases of cancer of the skin from the University of Santo Tomas Hospital, North General Hospital, and the Philippine Cancer Society in Manila revealed that 23.3 to 24.5 percent of the patients were at ages ranging from 40 to 50 and 50 to 60, respectively, (table 2). Another group of 17.5 percent of the total cases were from 60 to 70 years of age. This may be attributed to drying of skin with advancing age and degenerative changes which make it more susceptible to environmental trauma. In addition, the sum stimuli affecting tissues and organs increase with age (*3*). Xeroderma pigmentosum was found in the young.

TABLE 2.—Age incidence of skin cancers observed in 3 institutions*

Age group	UST (1953–59) 7 years	NGH (1946–60) 15 years	PCS (1958–61) 3 years	Total	Percent
1–10	0	0	0	0	0
11–20	5	7	4	16	4. 84
21–30	14	2	4	20	6. 06
31–40	22	12	5	39	11. 87
41–50	35	38	8	81	24. 54
51–60	19	50	8	77	23. 33
61–70	13	33	12	58	17. 57
71–80	1	23	6	30	9. 09
81–90	0	9	0	9	2. 73
Total	109	174	47	330	100

*UST = University of Santo Tomas; NGH = North General Hospital; PCS = Philippine Cancer Society, Inc.

Observations on the incidence of cancer by sex showed interesting findings. More men suffered from epidermoid carcinoma than did women (table 3). On the other hand, 61.5 percent of basal-cell carcinoma was found in women against 38.4 percent in men. In the Philippines, both men and women do outside work and therefore are subject to the same chronicity of exposure to many carcinogens. Melanomas were more frequent in males, representing 70.7 percent of the cases. The prevalence of cutaneous malignancy in men is worldwide. Roffo found the incidence of epithelioma in men to be 70.9 percent as compared to 29.1 percent in women (*3*).

LOCATION OF HISTOLOGIC TYPES OF SKIN CANCER

Epidermoid carcinomas were the most common. Of the 180 cases reported, 67 percent were in the head and neck. A considerable number were in the lower trunk and genitalia, probably due to the frequency of chronic inflammation in these areas (table 4).

As usual, basal-cell carcinomas were diagnosed on the head and neck in 92.3 percent of 104 cases. They were seldom found on the extremities and trunk. Melanomas were observed in 75 percent of 41 cases in the lower extremities. Adnexal carcinomas were rare and occurred in the head and neck.

In general, our observations agreed with reports of other authors that most skin cancers are found on exposed areas of the skin of the head and neck. A total of 223 malignant tumors in our series were at this anatomical site (text-fig. 3).

Table 3.—Incidence of skin cancers, by sex, observed in three institutions*

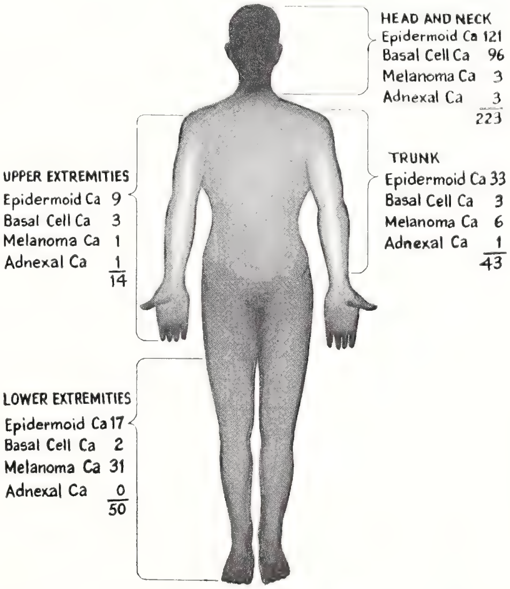
Histologic type	UST (1953–59) 7 years			NGH (1946–60) 15 years			PCS (1958–61) 3 years			Total for the 3 institutions				
											Males		Females	
	Total	Males	Females	Total	Males	Females	Total	Males	Females	Total	Number	Percent	Number	Percent
Epidermoid carcinoma	57	35	22	111	59	52	12	9	3	180	103	57. 22	77	42. 78
Basal-cell carcinoma	29	9	20	49	21	28	26	10	16	104	40	38. 46	64	61. 54
Melanoma	21	15	6	12	9	3	8	5	3	41	29	70. 73	12	29. 27
Adnexal carcinoma	2	1	1	2	0	2	1	1		5	2	40. 00	3	60. 00
Total	109	60	49	174	89	85	47	25	22	330	174	52. 73	156	47. 27

*UST = University of Santo Tomas; NGH = North General Hospital; PCS = Philippine Cancer Society, Inc.

TABLE 4.—Frequent types and sites of skin cancer among Filipinos*

Histologic type	UST (1953–59) 7 years	NGH (1946–60) 15 years	PCS (1958–61) 3 years	Total	
				Number	Percent
Epidermoid carcinoma	57	111	12	180	
Site:					
Head and neck	46	66	9	121	67. 22
Upper extremity	2	7	0	9	5. 00
Lower extremity	4	11	2	17	9. 44
Trunk	5	27	1	33	18. 33
Basal-cell carcinoma	29	49	26	104	
Site:					
Head and neck	26	45	25	96	92. 31
Upper extremity	2	0	1	3	2. 88
Lower extremity	1	1	0	2	1. 92
Trunk	0	3	0	3	2. 88
Melanoma	21	12	8	41	
Site:					
Head and neck	1	1	1	3	7. 32
Upper extremity	1	0	0	1	2. 43
Lower extremity	18	9	4	31	75. 61
Trunk	1	2	3	6	14. 63
Adnexal carcinoma	2	2	1	5	
Site:					
Head and neck	1	2	0	3	60. 00
Upper extremity	0	0	1	1	20. 00
Lower extremity	0	0	0	0	
Trunk	1	0	0	1	20. 00
Total skin cancers	109	174	47	330	

*UST = University of Santo Tomas; NGH = North General Hospital; PCS = Philippine Cancer Society, Inc.


HEAD AND NECK
Epidermoid Ca 121
Basal Cell Ca 96
Melanoma Ca 3
Adnexal Ca 3
223
TRUNK
Epidermoid Ca 33
Basal Cell Ca 3
Melanoma Ca 6
Adnexal Ca 1
43
UPPER EXTREMITIES
Epidermoid Ca 9
Basal Cell Ca 3
Melanoma Ca 1
Adnexal Ca 1
14
LOWER EXTREMITIES
Epidermoid Ca 17
Basal Cell Ca 2
Melanoma Ca 31
Adnexal Ca 0
50

TEXT-FIGURE 3.—Cancer of the skin by regional sites. From records of 330 cases from the University of Santo Tomas Hospital, North General Hospital, and the Philippine Cancer Society, Inc.

PATHOLOGICAL FEATURES

We divided the 330 malignant skin tumors into the following groups:

1) *Epidermoid carcinoma*—arising in the acanthotic area of the epidermis or prickle-cell layer. A total of 180 tumors were of this histologic type, and 121 were situated in the head and neck. They appeared as warty, ulcerating, papillary or cauliflower tumors in the lips, cheeks, dorsum of the hands, penis, vulva, buttocks, and in the temporal, preauricular, and malar regions. When patients with this type of tumor came to the clinic they usually were in the advanced stage, with extensive necrosis, serosanguinous discharge, and secondary infection. Most of these patients were treated as if they had infectious diseases. Curiously enough, only a small number of tumors metastasized to the regional lymph nodes. Histologically, 90 percent were classified as Grade I and II and only 10 percent as Grade III. A few of the spindle-cell type described by Stewart were found and showed rapid evolution. The squamous carcinomas arising from the lips, glans penis, and vulva metastasized early.

2) *Basal-cell carcinoma*—originating in the basal-cell layer or the dermal adnexae. Of the 104 tumors of this variety, 92 percent were located in the nose, nasolabial folds, eyelids, forehead, and ears. They appeared in these areas as small, shiny, reddish-yellow nodules with telangiectatic borders. The other lesions were typical rodent ulcers with pigmented, firm crater beds and rugged, irregular borders. They were often destructive but slow-growing. Not one tumor in this series metastasized. The medical practitioners often diagnosed these lesions as moles or melanomas. Microscopically, basal-cell carcinomas appeared as palisading polygonal cells with oval nuclei infiltrating into the dermis. Multiple foci or multicentric origin were demonstrated in a few instances. Cystic changes and melanin pigments were noted in a large number of cases.

3) *Malignant melanomas*—developing from benign nevi or from apparently normal skin. Of the 41 cases in the group, 75 percent were seen in the lower extremities—especially in the foot. They appeared as pigmented and ulcerated new growths with broad or pedunculated bases. Satellite blackish nodules were found in the periphery of the mother tumor. In most instances, metastases were noted before diagnosis could be established. On microscopic examination, these skin tumors were composed of malignant intraepidermal melanoblasts showing anaplasia and invasion of the deeper soft tissues. Junctional changes were demonstrated in a great number of cases. The juvenile melanomas were excluded in this group and were considered benign.

4) *Adnexal carcinomas*—originating in the sweat and sebaceous glands. We found 5 of these tumors; 3 were in the forehead and scalp (fig. 4). They were slow-growing, deeply invasive, and recurred after excision.

5) *Other rare skin cancers*—observed in several general hospitals. They were as follows: xeroderma pigmentosum—2 cases; mycosis fungoides—2 cases; Kaposi's hemorrhagic sarcoma—1 case; leukemia cutis—3 cases;

dermatofibrosarcoma protuberans—5 cases; and multiple neurofibromatosis with sarcomatous degeneration—1 case.

These varieties of skin cancer were not included in the analysis of the 330 cases, but they occurred in Filipinos. The case of mycosis fungoides, shown in figure 6, was mistaken for leprosy and the patient was confined in a leprosarium.

ETIOLOGY

In tracing the etiopathogenesis of our reported cases, we relied on data submitted from the record sections of the 3 institutions and on questionnaires sent to medical practitioners, mining companies, and industrial firms. The following potential causes were considered: exposure to sunlight; trauma; burns; ultraviolet rays; X rays; chemicals; infections; dietary habits; occupation; and heredity (*3*, *4*).

That solar radiation has carcinogenic properties is substantiated by the arguments of Roffo that malignancy of the skin appears most frequently in parts of the body exposed to sunlight and may be produced in experimental laboratory animals. He added further that there is a direct relationship between cholesterol content of the skin in the incidence of malignancy. In the opinion of many, the radiant energy of the solar spectrum and carcinogenic hydrocarbons added together produce skin cancer. Age of the individual is not a factor in solar epithelioma. The only time factor is length and degree of specific exposure (*5*). Solar hyperkeratosis of the face and other exposed regions of the body are precancerous. In our series of 330 cancer cases, 67 percent were in the exposed areas of the head and neck and upper extremities. In these groups, the patients were farmers, fishermen, sailors, policemen, and marine engineers (fig. 1). Women have as high an incidence of skin cancer as men because they are equally exposed to the same solar factors in the rice fields and at the river banks, where most laundry women do their washing (fig. 2). We have also seen malignant skin neoplasms among street sweepers who are similarly exposed.

Repeated trauma and irritation played a definite role in 15 cases of malignant melanoma. Three Filipino houseboys, who polished floors with coconut husks, developed this type of skin tumor on the plantar surface of their feet (fig. 5). A young boy developed cancer of the skin after severe burns (fig. 7). An unusual case of epidermoid carcinoma developed in the leg of an old man after years of using a Japanese magnetic bracelet for treatment of arthritis and hypertension. Squamous carcinomas developed in 3 patients whose feet were irradiated for athlete's foot after a lag period of from 10 to 15 years of radiation keratosis (fig. 8).

We never observed malignancies of the skin due to crude tar, paraffin, arsenic, benzol, and other chemicals. Many physicians at mining companies, tanneries, and oil and chemical factories that were interviewed did not report 1 case of skin cancer. Perhaps this is because the Philippines is in its early period of industrialization. Moreover, due to the preventive measures instituted by company physicians, the workers are less exposed to chemical carcinogens. There were numerous cases of contact dermatitis reported but not a single case of cancer.

The theory of heredity and light as causative factors was verified in the xeroderma pigmentosa. The concept that cancer can be produced by faulty food habits and parasites was not borne out by the histories of these patients. We never saw epidermoid carcinomas arising in such infectious lesions as tropical ulcers, yaws, lupus vulgaris, osteomyelitis sinuses, or scars from smallpox vaccination. With modern antibiotic therapy these infections were controlled. However, an old lady developed epidermoid carcinoma in a large sinus of the skin in the lower abdomen because of marsupialization of a cystic ovary for 20 years. Four of our cases revealed squamous carcinoma in the prepuce possibly due to poor penile hygiene or noncircumcision.

In the Chinese General Hospital we saw in 7 years only 3 cases of skin cancer out of 5,000 surgical specimens. It is possible that because of the sheltered lives of Chinese businessmen and storekeepers in the Philippines they are less exposed to the sun. Yet we have discovered many skin cancers among Americans and European missionary priests and businessmen staying in the country for many years. Racial predisposition may be important. Besides, blondes and those with fair skin are susceptible to chemicals and radiant energy (*3, 4*). The possible factors for these differences need more comprehensive epidemiological investigation.

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PLATES

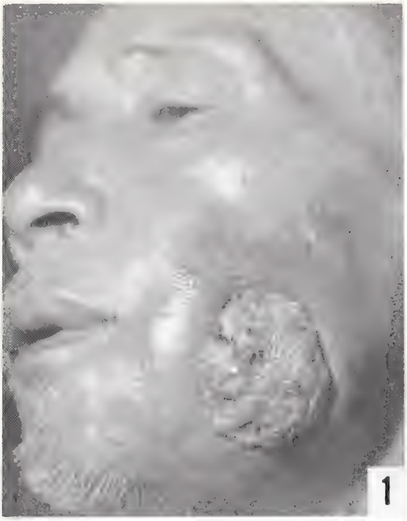
PLATE 8

FIGURE 1.—Fungating epidermoid carcinoma in an old farmer.

FIGURE 2.—Ulcerating epidermoid carcinoma in a laundry woman.

FIGURE 3.—Basal-cell carcinoma aggravated by years of ultraviolet application.

FIGURE 4.—Adnexal carcinoma of the scalp.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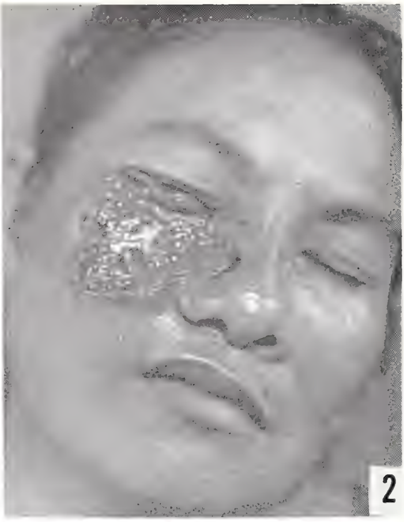
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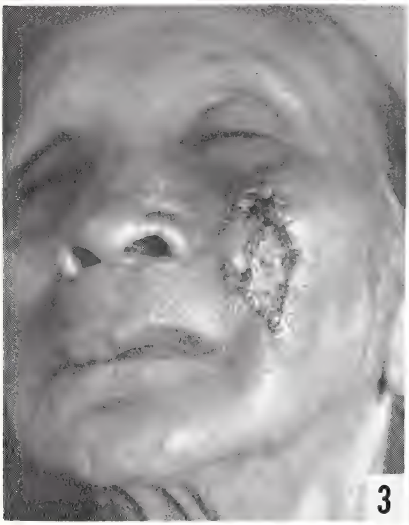
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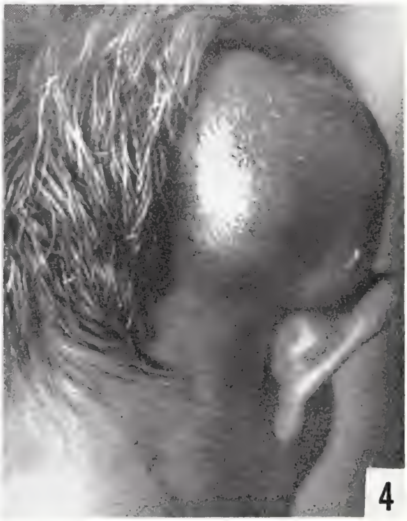
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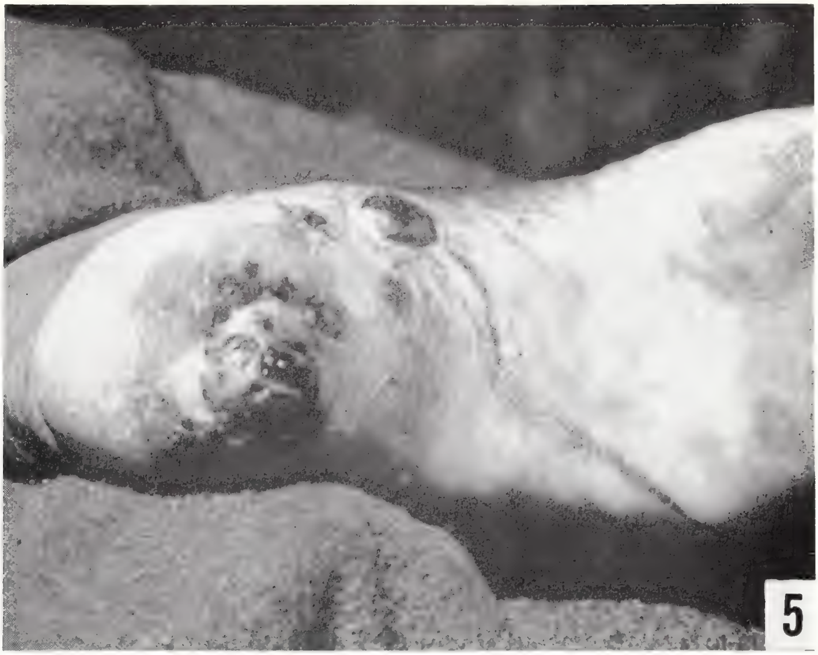
PANTANGCO, CANLAS, BASA, AND SIN

PLATE 9

FIGURE 5.—Malignant melanoma on the heel of the foot of a houseboy.

FIGURE 6.—Mycosis fungoides mistaken for leprosy.



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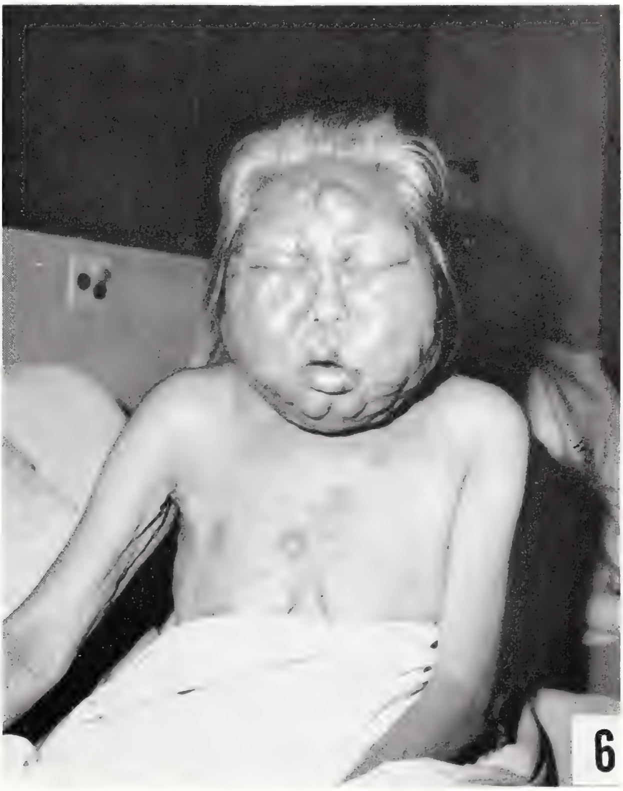

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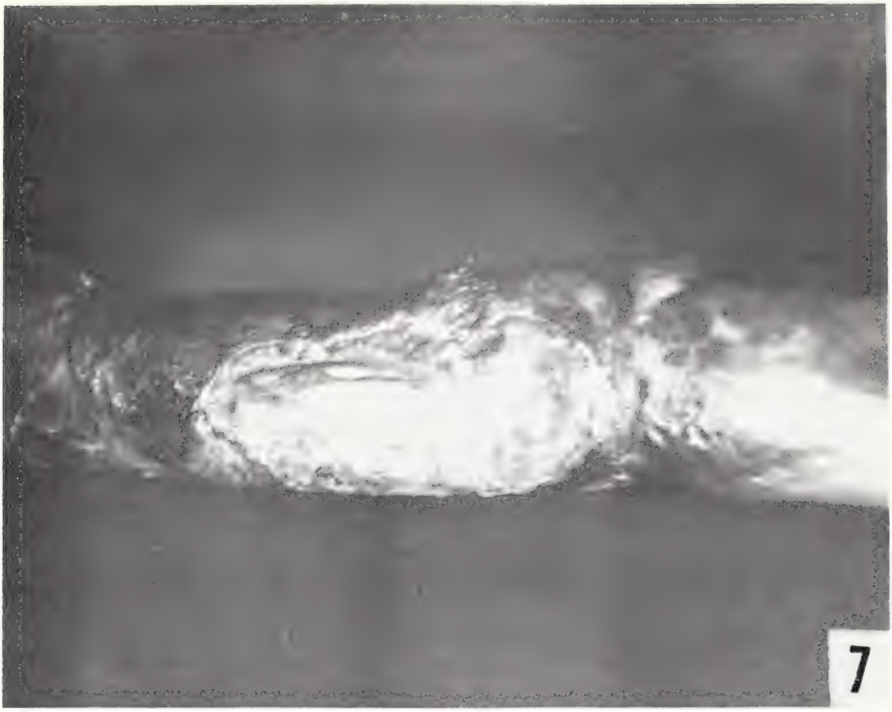
PLATE 10

FIGURE 7.—Squamous-cell carcinoma secondary to keloid after severe burns.

FIGURE 8.—Squamous-cell carcinoma degenerating from radiation keratosis.



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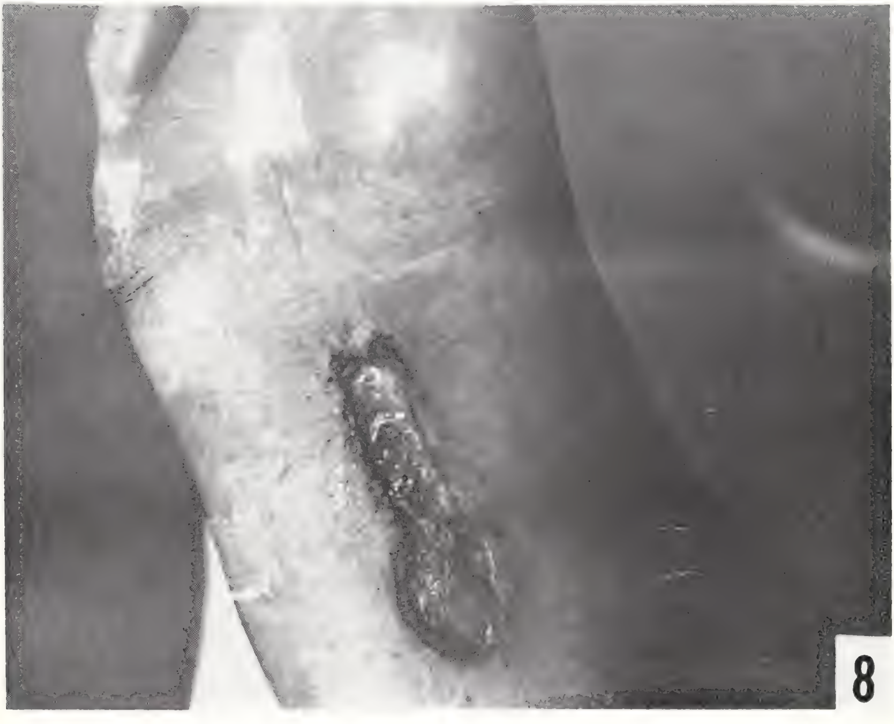

8

SKIN CANCER IN SINGAPORE[1]

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SUMMARY

The population of Singapore comprises Chinese, Malaysians, Indians, Pakistanis, and others. The limitations of the available morbidity and mortality figures are discussed. Four hundred ninety-three cases of cancer arising on the body surface were diagnosed by biopsy during a 12-year period (1950–61): skin 352, melanoma 32, lip 18, vulva 20, penis 65, and scrotum 6. Melanomas occurred mostly on the feet and appeared to be less frequent in Singapore than in the less urbanized neighboring territories. Skin cancers were relatively more frequent among the Caucasoids than in the more pigmented Asian races; the more deeply pigmented Indians were the least prone to develop cancer on the exposed areas of the skin. Squamous-cell carcinomas outnumbered basal-cell carcinomas in the Asian races as a result of an increased frequency of squamous-cell carcinomas on the legs and feet as well as a reduced frequency of basal-cell carcinoma, which corresponded with the depth of skin pigmentation in the various race groups. Racial variations in the site distribution of the neoplasms are discussed. There was no significant difference between the frequencies of penile carcinomas in the Malays, who are usually circumcised when they are 12 to 14 years old and in the noncircumcised Chinese and Indians.—NCI Monogr. No. 10: 127–140, 1963.

THE ISLAND of Singapore has certain distinctive characteristics that are well suited for the observation of the influence of inherited and environmental factors on the pattern of skin cancer. The climate is hot (average temperature 85°), moist (average humidity 83%), and equable, and there is bright sunlight throughout the year. The population of approximately 1½ million has a multiracial and predominantly Asian composition and comprises the indigenous people who were born in Singapore as well as immigrants who constitute two thirds of the population above 30 years of age. Fifty-two percent of the total population is under the age of 20. The Chinese (73% of the adult population) are for the most part derived from the southern provinces of China, the brown-skinned Malaysians (13%) are the natives of the country, the majority of the

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia Penna., April 6–11, 1962.

[2] The authors wish to thank Dr. A. O. Aaron, Pathologist, and Dr. Ho Yuen, Medical Superintendent, General Hospital, for use of records, and Dr. Ng See Yook, Director of Medical Services, for permission to publish; and to express their appreciation to the technicians in the Government Department of Pathology for the histological preparations, to Mr. I. Nadarajah, Records Officer, for information on hospital admissions, to Miss Lucy Leow for clerical assistance, to Mr. Chew Kiat Seng for the preparation of the text-figures, and to Mr. P. A. Samuel for typing the script.

Indians and Pakistanis (11%) are the dark-skinned Tamils from South India, and the others (3%) belong to a variety of races of whom a majority are Caucasoids (1.1%) and Eurasians (0.9%). Consequently, there is a wide range of skin pigmentation, and the modes of life are changing rapidly due to increasing urbanization and cultural exchange among the people. In this paper we shall review briefly some of the available information on the morbidity and mortality of skin cancer and present a more detailed analysis based on the records of the Singapore Pathology Department.

MORBIDITY

The *Annual Reports of the Singapore Medical Department* show that skin cancer accounts for approximately 1.5 percent of the total morbidity from cancer (table 1). However, this figure refers only to hospital admissions, and, as a fair proportion of persons with skin cancers are treated as outpatients, it minimizes the proportion of skin cancer in relation to cancers from other sites. Reports of clinical experience have been influenced by the specialty of the observer. For example, Khoo (*1*) reported that skin cancer was definitely rare among the domiciled races of Singapore, but the fact that he did not find a single case of skin cancer

TABLE 1.—Morbidity and mortality of cancers of the skin (International List Nos. 190, 191) in Singapore and in certain selected countries

		Singapore	Japan	Australia	England and Wales	U.S. whites	U.S. nonwhites
Morbidity as a % of all cancers		1. 5*			13. 4†		
Mortality as a % of all cancers		2. 1*	0. 7‡	2. 5‡	1. 0‡	1. 6‡	0. 7‡
Age-standardized death rates per million per annum for the age group 35–64	Males	47. 4§	11. 1‖	42. 7‖	16. 8‖	33. 1‖	18. 6‖
	Females	10. 5§	8. 7‖	27. 9‖	15. 7‖	21. 3‖	12. 0‖
Age-standardized death rates per million per annum for the age group 65+	Males	32. 7§	78. 3‖	278. 5‖	155. 8‖	181. 7‖	63. 4‖
	Females	94. 3§	54. 7‖	154. 3‖	90. 0‖	111. 2‖	45. 2‖
Mortality rates per million per annum for melanoma (List No. 190)		1. 0§	0. 3¶	22. 0¶	8. 7¶	12. 4¶	2. 6¶
Mortality rates per million per annum for other cancers of the skin (List No. 191)		7. 7§	6. 4¶	13. 5¶	12. 7¶	12. 1¶	5. 9¶

*1954–58 (*3*); †1957 (*6*); ‡1952–56 (*5*); §1957–58 (*3*); ‖1952–56 (*7*); ¶1957 (*8*).

among 1,000 consecutive patients seen at the Skin Clinic of the General Hospital was probably because cancer patients were often referred directly to the surgical and radiotherapy units. A total of 144 patients with skin cancer were treated at the Radiotherapy Department of the General Hospital in 7 years [1955–61 (*2*)]. Fifty-three had squamous-cell carcinomas and 91 had basal-cell carcinomas, but this distribution would have been influenced by the selection of patients for radiotherapy. A more representative and comprehensive picture of the disease pattern is provided in the analysis of histological examinations.

MORTALITY

The mortality rates per million per annum (*3*) have been estimated to be 1.0 for melanoma (International List No. 190) and 7.7 for other cancers of the skin (List No. 191). However, that more than half the population is below the age of 20 and that only two thirds of all deaths are certified by qualified practitioners should be considered in the interpretation of these figures. The mortality from skin cancer expressed as a percentage of all cancers is comparatively high. Muir derived the figures in table 1 from an analysis of the *Annual Reports of the Ministry of Health and the Registrar-General* (*3*). Using the formula of Stocks (*4*), Muir calculated the age-standardized death rate for skin cancer (List Nos. 190, 191) per million per annum for the age group 35 to 64 to be 47.4 percent for males and 10.5 percent for females. He points out, however, that these figures are much higher than the actual, in view of the differences between the population structures of Singapore and of England and Wales in respect to the relative proportions of the populations in the 3 decades comprising this age group. The reasons for the surprisingly low rates for the age group 65+ are not clear. It is possible that deaths among older people are certified relatively more frequently by unqualified persons, and it is also likely that the older people, an overwhelming majority of whom are immigrants, may return and die in their countries of origin. The figures from the Ministry of Health and the Registrar-General, therefore, do not provide a reliable means of comparing the skin cancer incidence in Singapore with that of other countries.

Necropsies

Only 6 cases of skin cancer were observed in 12 years (1947–58), and the neoplasm did not rank within the first 20 primary sites established at necropsy. Necropsy figures belittle the frequency of skin cancers since only a few of these patients die in a hospital and since elective necropsies are performed less often in the absence of diagnostic difficulties; skin cancers are easily accessible for biopsy and are usually diagnosed during life.

BIOPSIES

The Department of Pathology provides a histological service for the whole island and receives specimens from all hospitals and clinics as well as from private practitioners. The histological examinations made elsewhere are so few that for practical purposes the records of the Department of Pathology, which are analyzed here, may be regarded as a representation of all histologically diagnosed cases among the Singapore population. During a 12-year period (1950–61), 42 malignant melanomas (List No. 190) and 583 other skin cancers (List No. 191) were found among 69,978 tissues from 61,357 examinations. These figures include duplicate examinations on the same case and provide no more than a rough estimate that skin cancer forms about 6 percent of histologically diagnosed cancers, ranking third in frequency after the cervix uteri and the nasopharynx. The accessibility of skin cancers for biopsy tends to exaggerate their frequency among histologically diagnosed cases.

The exclusion of all duplicate examinations and 4 cases with inadequate information left 352 cases of skin cancer and 32 cases of melanoma, which are analyzed, together with cancers of the lip (18), vulva (20), penis (65), and scrotum (6)—a total of 493 separate cases of cancer arising on the body surface during 1950–61. The race and sex distribution of these patients are shown in table 2; the site distribution for skin cancers in table 3, and the age distribution for all races and for the Chinese in tables 4 and 5

Malignant Melanoma

The primary sites in the 32 cases of malignant melanoma in this series were as follows: feet 22, head and neck 4, trunk 2, and anus, vulva, thumb, and knee 1 each.

In contrast to the site distribution in Caucasoids, in whom the majority of melanomas are found in the head and neck (*9*) and in whom the possible influence of sunlight has been postulated (*10*), the tumors in Asian countries such as Malaya (*11*), Ceylon (*12*), and Java (*13*), are predominantly distributed on the feet. Trauma has been widely accepted as the most important environmental factor in Asian countries where melanomas of the feet are frequently associated with the barefoot habit. The possible influence of an independent genetic factor is suggested by the scarcity of tumors on the head and neck and the exceptional rarity of melanomas of the eye and uveal tract in Malaya (*11*) and Singapore.

The ratio of 32 melanomas to 352 other cancers of the skin (0.09) is low in comparison with Caucasian populations. In the neighboring territory of Malaya, however, Marsden (*11*) found a ratio of 85 malignant melanomas to 400 other skin cancers (0.21) and commented on the especially high incidence of melanomas among the Indians. In his series the ratios of malignant melanomas to other skin cancers were 22 : 168 (0.13) in Malays, 36 : 178 (0.20) in Chinese, and 27 : 54 (0.50) in Indians: two thirds of all melanomas were in the feet. Our ratios in Singapore

Table 2.—Race and sex distribution of patients with cancers arising on the body surface

Cancers (International List Nos.)	Chinese			Indians and Pakistanis			Malays			Others			All races		
	M	F	Total	M	F	Total	M	F	Total	M	F	Total	M	F	Grand total
Lip (140)	5	1	6	6	1	7	—	2	2	3	—	3	14	4	18
Vulva (176.0)	—	17	17	—	—	—	—	2	2	—	1	1	—	20	20
Penis (179.0)	46	—	46	14	—	14	5	—	5	—	—	—	65	—	65
Scrotum (179.1)	3	—	3	2	—	2	1	—	1	—	—	—	6	—	6
Skin—melanoma (190)	16	14	30	1	—	1	1	—	1	—	—	—	18	14	32
Skin—other cancers (191)															
Squamous-cell carcinoma	88	39	127	14	3	17	14	3	17	8	5	13	124	50	174
Basal-cell carcinoma	58	42	100	4	—	4	6	6	12	39	9	48	107	57	164
Adnexal carcinoma	5	7	12	1	—	1	1	—	1	—	—	—	7	7	14
Total	221	120	341	42	4	46	28	13	41	50	15	65	341	152	493
% All biopsies			79. 2			10. 7			5. 4			4. 7			100. 0
% Hospital admissions*	46. 1	28. 2	74. 3	11. 5	3. 6	15. 1	4. 9	2. 4	7. 3	2. 2	1. 0	3. 2	64. 7	35. 2	99. 9
% Outpatient attendance†	37. 9	29. 8	67. 7	15. 5	5. 2	20. 7	5. 3	2. 8	8. 1	2. 2	1. 2	3. 4	60. 9	39. 0	99. 9
% Population above 20 years‡	36. 4	36. 6	73. 0	8. 6	2. 2	10. 8	7. 3	5. 9	13. 2	1. 6	1. 4	3. 0	53. 9	46. 1	100. 0

*Records of the General Hospital, Singapore, for 1960 and 1961.

†Derived from the *Annual Reports of the Ministry of Health, 1956-58.* Children below 12 years excluded.

‡Derived from the *1957 Census of Population, Preliminary Release No. 5,* Govt. Printing Office, Singapore 1959.

TABLE 3.—Site distribution of cancers of the skin (International List No. 191)

Type and site	Chinese			Indians and Pakistanis			Malays			Others			All races		
	M	F	Total	M	F	Total	M	F	Total	M	F	Total	M	F	Grand total
Squamous-cell carcinoma															
Head and neck	32	21	53	3	2	5	2	1	3	4	3	7	41	27	68
Upper limbs	9	1	10	—	—	—	2	—	2	3	1	4	14	2	16
Lower limbs	28	6	34	2	1	3	7	1	8	1	—	1	38	8	46
Trunk	19	11	30	9	—	9	3	1	4	—	1	1	31	13	44
Basal-cell carcinoma															
Forehead and scalp	3	3	6	1	—	1	—	2	2	9	2	11	13	7	20
Eyelids	10	6	16	1	—	1	3	1	4	2	1	3	16	8	24
Nose	20	15	35	—	—	—	—	2	2	5	5	10	25	22	47
Elsewhere on face	16	12	28	2	—	2	3	1	4	11	1	12	32	14	46
Elsewhere on body	8	4	12	—	—	—	—	—	—	9	—	9	17	4	21
Unknown	1	2	3	—	—	—	—	—	—	3	—	3	4	2	6
Adnexal carcinoma	5	7	12	1	—	1	1	—	1	—	—	—	7	7	14
Total	151	88	239	19	3	22	21	9	30	47	14	61	238	114	352

Table 4.—Age distribution of cancers in all races—site, type, and sex

International List Nos.	176.0	179.0	179.1	140		191												190	
						Squamous-cell carcinoma													
				Lip		Head and neck		Upper limbs		Lower limbs		Trunk		Basal-cell carcinoma		Adnexal carcinoma		Melanoma	
Age groups	Vulva	Penis	Scrotum	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
0–9															2				
10–19											1				1				
20–29	1	3				2	1			2		1		1	2		1	3	1
30–39	6	10	1	2		2	3			4		7	1	11	4				2
40–49	5	13	2	6		6	4	3		4		4	4	24	16	1	5	5	1
50–59	5	25		5	1	13	4	4		13	3	9	3	35	6	2		5	6
60–69	3	9	1		2	6	5	5	2	10	3	7	4	23	10	3	1	3	3
70–79		3	2	1	1	11	9	1		5	1	3	1	7	10	1		1	1
80+						1	1	1							3			1	
Unknown		2												6	3				
Total	20	65	6	14	4	41	27	14	2	38	8	31	13	107	57	7	7	18	14

Table 5.—Age distribution of cancers in Chinese—site, type, and sex

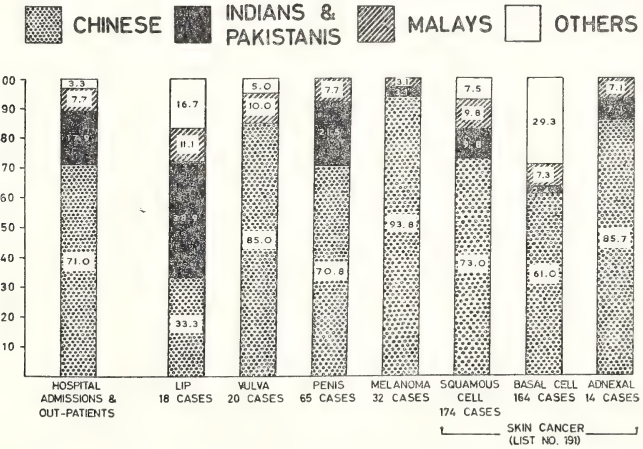
International List Nos.	176.0	179.0	179.1	140		191												190	
						Squamous-cell carcinoma													
				Lip		Head and neck		Upper limbs		Lower limbs		Trunk		Basal-cell carcinoma		Adnexal carcinoma		Melanoma	
Age groups	Vulva	Penis	Scrotum	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
0–9															2				
10–19											1				1				
20–29	1	1								1				1			1	3	1
30–39	6	4		1		1	1			2		4	1	6	3				2
40–49	3	11	1	1		6	4	3		1		3	4	9	13	1	5	4	1
50–59	4	19		2		10	4	1		11	3	6	2	19	5	2		4	6
60–69	3	7				4	4	3	1	9	1	4	4	18	7	1	1	3	3
70–79		3	2	1	1	10	8	1		4	1	2		5	7	1		1	1
80+						1		1							3			1	
Unknown		1													1				
Total	17	46	3	5	1	32	21	9	1	28	6	19	11	58	42	5	7	16	14

were 1 : 30 (0.03) in Malays, 1 : 22 (0.05) in Indians, and 30 : 239 (0.12) in Chinese. The higher ratio of melanomas in Malaya than in Singapore, especially among the Indians and Malays, may be related to the predominantly rural population of Malaya, including the barefoot laborers in the rubber estates and padi fields, in contrast to the more urbanized population of Singapore. The situation may well be analagous to the observed high incidence in some African populations (*14*, *15*) and the low frequency among the Negroes of America (*16*).

Skin Cancer (International List No. 191)

There were 352 skin cancers in this series: 174 squamous-cell carcinomas, 164 basal-cell carcinomas, and 14 carcinomas of adnexal origin. Reports from China (*17–19*), Ceylon (*12*), and Malaya (*11*) have shown that skin cancers occupy a high rank among cancers diagnosed histologically in these countries. The relative proportion of skin cancers among other histologically diagnosed cancers in Singapore is lower than that in Malaya, and it is tempting to attribute this to the greater degree of urbanization and the higher standard of living and of medical care in Singapore. But such a relationship cannot be postulated until a reduced morbidity is established by means of rate surveys.

Squamous-cell and basal-cell carcinomas were relatively more frequent among the "others" than among the Chinese, Indians, and Malays (text-fig. 1). Of the 61 skin cancers occurring in the races grouped under "others," 52 were in Caucasoids (who formed only one third of this group), 7 were in Eurasians, 1 in a Jew, and 1 in a Siamese. An overwhelming majority of the patients in this series was seen at the General Hospital and comprised approximately equal numbers of outpatients and inpatients. Hence in text-figure 1 the race distribution of cancer patients is compared



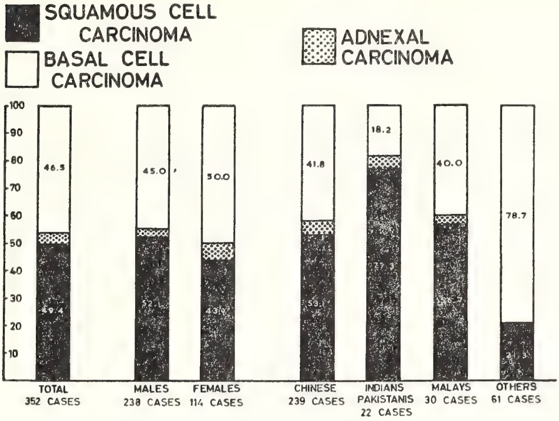
TEXT-FIGURE 1.—Race distribution of cancers arising on all parts of body surface, excluding the scrotum.

with that of the mean of hospital admissions and outpatients. The race distribution of the population was considered unsuitable for comparison because of racial variations in willingness to seek hospital treatment and, since there were large numbers of gynecological specimens, histological examinations were considered unsuitable. Both of these have been presented in table 2. The Caucasoids formed only 0.9 percent of hospital patients but accounted for 14.8 percent of skin cancers. The Eurasians constituted 1.3 percent of hospital patients and accounted for 2.0 percent of skin cancers. The corresponding figures for the Chinese were 71.0 and 67.9 percent, for the Indians and Pakistanis 17.9 and 6.2 percent, and for the Malays 7.7 and 8.5 percent. As other types of cancer, such as those arising in the cervix uteri, liver, lung, gastrointestinal tract, and nasopharynx, have been found to be relatively infrequent among the Caucasoids in Singapore, the figures mentioned provide convincing evidence that the Caucasoids are more prone to develop skin cancer than the more pigmented races of Singapore. The Indians who are the most deeply pigmented group in Singapore appear to be the least prone to develop cancer in the exposed areas of the skin.

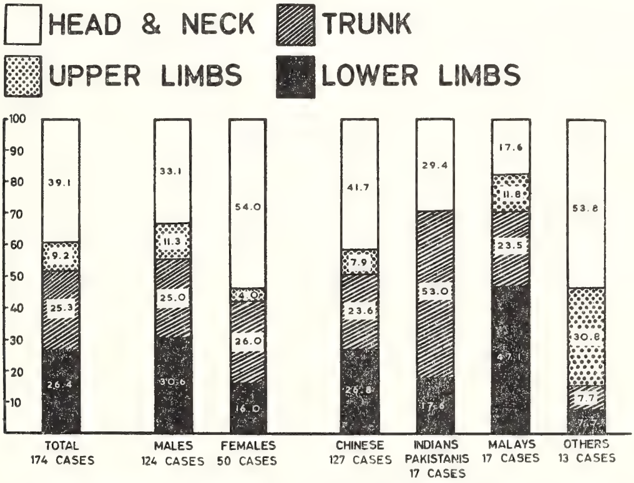
In contrast to the pattern in Caucasoids, squamous-cell carcinomas have outnumbered basal-cell carcinomas in Africa (*15*, *16*, *20*), China (*19*), Ceylon (*12*), Thailand (*21*), Malaya (*11*), and in the present series in Singapore. Although rates for different types of skin cancer are not available, it is evident that this reversal of the squamous-cell : basal-cell ratio is the outcome of both an increased frequency of squamous-cell carcinoma in certain sites, *e.g.*, the legs and feet where carcinoma is uncommon in Caucasoids as well as a reduced frequency of basal-cell carcinoma. In the present series, the squamous-cell : basal-cell ratio in the various race groups corresponded with depth of skin pigmentation, being 17:4 (4.3) for Indians, 17:12 (1.4) for Malays, 127:100 (1.3) for Chinese, and 13:48 (0.3) for "others"; the ratio for the Caucasoids in the last group was 9:43 (0.2). If squamous-cell carcinomas arising in the legs and feet were removed from the total number, basal-cell carcinomas would outnumber the squamous in all races except the Indians. The reduced frequency of basal-cell carcinoma, which has also contributed to the reversal of the squamous-cell: basal-cell ratio, is partly attributable to the relative youth of the population, but the scarcity of the tumor in the more deeply pigmented Indians and the corresponding increase in frequency with decreasing pigmentation in the Malays, Chinese, and others in Singapore lends strong support to the protective influence of skin pigmentation on the susceptibility to develop basal-cell carcinoma (text-fig. 2).

Squamous-Cell Carcinoma

The sites of occurrence of these tumors in descending order of frequency were the head and neck, lower limbs, trunk, and upper limbs. The first ranking site in the Chinese was the head and neck followed by the lower limbs and the trunk; in the Indians it was the trunk; in the Malays the



Text-figure 2.—Ratios of squamous-cell, basal-cell, and adnexal carcinomas of the skin by race and sex.



Text-figure 3.—Site distribution of squamous-cell carcinomas of skin by race and sex.

lower limbs; and in the "others" it was the head and neck followed by the upper limbs (text-fig. 3).

The available clinical histories were insufficient to allow an examination of the possible factors influencing these site variations, but it is interesting to note that in Malaya (*11*) the skin cancers in the Malays, who are the least urbanized section of the population, were likewise situated mainly in the lower limbs. Squamous-cell carcinomas of the skin were relatively infrequent among the Indians, but an examination of the site distribution (table 3) shows that whereas among the Indians there were only 8 (6.2%) of 130 squamous-cell carcinomas occurring in the more exposed areas, such as the head and neck, upper and lower limbs, 9 (20.5%) of the 44 tumors occurred in the trunk. The high proportion of trunk carcinomas among the Indians is due to an increased frequency in this site as well as a reduced frequency in other areas. However, skin cancers arising in the trunk were situated mainly in the anoperineal region and the groin and it is interesting that Khanolkar and Suryabai (*22*) have described similar tumors following "dhoti" irritation among the Indians in Bombay. The Indians in Singapore are mostly Tamils who do not wear such "dhotis" but some of them wear the traditional loin cloths as undergarments; however, no information on the clothing habits of the patients in this series is available.

Basal-Cell Carcinoma

The race differences in frequency have been referred to previously. The majority of the tumors were in the Chinese and Caucasoids. In the Chinese there were relatively fewer tumors on the forehead and scalp than on the nose and other parts of the face, but there was no such site predilection in Caucasoids. Most of the cases were in the 5th, 6th, and 7th decades of life. There were 10 cases of multiple basal-cell carcinoma, 3 with trichoepitheliomatous features in young female Chinese aged below 20 years and 7 rodent carcinomas in adults—these were classified according to the site of first appearance or predominant distribution in table 3.

Adnexal Carcinoma

Eleven of the 14 adnexal carcinomas in this series were sebaceous carcinomas. These were distributed in the scalp (6), eyelids (3), and face (2). The neoplasms were slow-growing, but the scalp tumors frequently reached large dimensions.

Carcinoma of the Lip

Carcinomas of the lip were relatively more frequent among the "others" (all 3 cases in this group were Caucasoids) and the Indians. The cases in the Chinese were outnumbered by those in the smaller Indian population; this is probably related to the high incidence of buccal cancer among the Indians associated with their habit of betel chewing. Cancers of the lip and oral cavity have occurred with very high frequencies in India (*23*, *24*) and Ceylon (*12*). In Singapore, however, the cultural association of the Indians with the other communities has led to a decline in the habit of betel chewing and these cancers are seen less frequently. The 18 cases in the series were all well-differentiated squamous-cell carcinomas.

Carcinoma of the Vulva

There were 20 cases of vulval carcinoma, 17 in Chinese, 2 in Malays, and 1 in the race group "others" who was a Siamese. Two cases were associated with lymphogranuloma inguinale. There were 19 squamous-cell carcinomas arising from the epidermis and vestibular mucous membrane and 1 adenocarcinoma arising in Bartholin's gland.

Carcinoma of the Scrotum

Scrotal cancers numbered only 6 cases in this series and were consequently excluded from the analysis in text-figure 1. The tumors were well-differentiated squamous-cell carcinomas.

Carcinoma of the Penis

There were more squamous-cell carcinomas arising in the penis than in the head and neck, limbs, or trunk in males. The proportion, however, was not as high as those recorded in China (*17*, *19*, *25*), India (*26*), and Ceylon (*12*). In Malaya (*11*) and Indonesia (*27*) the frequency of penile cancer among the circumcised Malays and Indonesians has been reported to be lower than in the uncircumcised Indians and Chinese in these territories. In the present series, however, the number of penile cancers in Malays (5 of 65—7.7%) was roughly proportionate to the race distribution of hospital patients (text-fig. 1). In view of the established protective influence of circumcision, this finding is probably attributable to the fact that circumcision in Malays is usually delayed until 12 to 14; 1 of the Malays in this series, a young man aged 28, was uncircumcised.

The tumors are squamous-cell carcinomas and are so well differentiated that their distinction from papillomatous lesions and pseudoepitheliomatous hyperplasia may occasionally cause diagnostic difficulty.

Miscellaneous Lesions

There were 11 cases of *keratoacanthoma,* 4 in male Caucasoids, 4 in male Chinese, and 3 in female Chinese. The lesions were single and presented the characteristic microscopic structure and history of rapid growth of short duration. Six were situated on the head and neck, 4 on the chest wall, and 1 on the shoulder.

There were 5 cases of *Bowen's disease* and 9 of *senile keratosis and leukoplakia.* These lesions were situated in the head and neck in 7 cases, trunk in 3, upper limbs in 1, lower limbs in 2, and vulva in 1.

Superficial *sarcomas* with skin involvement were diagnosed in 14 cases, but were excluded from this series of skin cancers as they were probably extensions of neoplasms arising in the subcutaneous soft tissues and nerve sheaths.

Cutaneous metastases from distant neoplasms were found in 73 cases (45 males and 28 females). The primary site was established in only 20 cases: lymphoma (5), liver (4), pancreas (3), suprarenal and thyroid (2 each), and breast, bone, kidney, and choriocarcinoma (1 each). The remaining 53 comprised 10 adenocarcinomas, 9 squamous-cell carcinomas, and 3 undifferentiated neoplasms.

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EPIDEMIOLOGY OF SKIN CANCER IN NEW ZEALAND[1]

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SUMMARY

The incidence of malignant melanoma is high in New Zealand. It is much higher than in Britain, higher generally than in the United States, but not so high as in Australia. When considered by site, malignant melanoma of exposed parts of the body shows a pattern different from that of other sites. In the former, in both sexes, there is an exponential rise with increasing age. In the latter, the curves are humpbacked, with the modal point in women some 15 years earlier than the modal point in men. Taken together, these data suggest that an external factor(s), probably sunlight, may affect exposed sites, and that an internal factor(s) also operates, which may well be hormonal. In New Zealand, the proportion of malignant melanoma in exposed sites in humans is not greater, but appears less than, in some other countries with a much lower total prevalence. Exposure to sunlight may be one of several causative or provocative factors in the development of malignant melanoma of the skin, but it cannot *per se* explain the large differences that exist among Britain, Australia, and New Zealand. The significance of the distribution, by age, sex, and site, of skin cancers in New Zealand other than malignant melanoma, of the frequency of postulated antecedent factors, and of differences in all these points between New Zealand and other countries is discussed. Special comment is made on exposure to sunlight as the prime cause of skin cancer, and the relationship between such exposure and precise site distribution. It is suggested that precusory stromal changes cannot wholly explain this distribution. Also discussed are the distribution and activity of sebaceous glands and the possibility that the interaction of sunlight on fatty substances in the skin of these special sites induces carcinomatous changes. The teleological argument that man developed blondeness when he migrated to the sunless north, thus making his skin a more efficient producer of vitamin D, is pushed further to suggest that, in the process he laid himself open to the concomitant production by radiation of small quantities of carcinogen from related sterols. When the blonde man moved nearer the equator, production of these carcinogens became critical. On the basis of this hypothesis, some of the exceptionally high incidence of skin cancer in Australia and New Zealand may be due to a high intake of animal fats. A fortuitous finding was a high incidence of chronic lymphatic leukemia among males with skin cancer. Six were observed where 0.2 were expected.—NCI Monogr. No. 10: 141–151, 1963.

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TOPOGRAPHY OF NEW ZEALAND

New Zealand lies south of the equator between latitudes 34° S and 48° S, and its area includes two long, thin, rugged, mountainous, windswept islands over 800 miles from north to south, and several smaller outlying islands. Seventy percent of the population lives in the North Island, the smaller of the two, and three fifths of the population of that island lives in its northern half, north of the 39th parallel.

Auckland, the largest center of population, lies at latitude 37° S, and corresponds in the antipodes to a point near the Strait of Gibraltar, and were the whole country transposed to the Northern Hemisphere, the rest of it would extend upward and outward into the Atlantic Ocean until the southernmost tip of the South Island were almost on a level with Paris.

It is a comparatively sunny country. The occupied areas of the South Island are drier than those of the North Island so that the effect of farther distance from the equator is offset by longer hours of sunlight.

At the time of the last census in April, 1961, there were 2,174,000 people, of whom 137,000 described themselves as Maoris, that is they were half or more of Polynesian descent. Though we are a predominantly agricultural country, a majority of our people live in cities, most of which are located by the sea. Only 2 of our 15 urban areas, those with a population of 20,000 or more, are situated inland. Fifty-five percent of our population live in these areas. There are no very large cities such as exist in Australia; Auckland, the largest, has a population of 380,000.

The beach and the open country, which we call the bush, is easily accessible to everyone, and like the ancient Greeks we love the outdoor life—games, sports, swimming, climbing, fishing, hunting, or just picnics and sitting around outside. Even in the heart of our big cities practically every house has its own garden and the householder generally spends considerable time working in it.

Our industry is of comparatively recent origin, light and secondary in type, and modern in its methods, buildings, and equipment.

In a view of a number of statements that have been made about regional differences in the incidence of skin cancer in New Zealand, I should like to say at the outset that a careful assessment of all the data has failed to establish their validity, and when we consider the uniformity of the country, the small population involved, and the topographical details already adumbrated, this is not surprising. Though such differences may exist they cannot be proved on the basis of existing data.

The comparisons made are therefore limited to those between New Zealand and other countries, with all the difficulties and uncertainties that such international comparisons inevitably involve.

SOURCE OF DATA

We have had an efficient nation-wide Cancer Registration Scheme in New Zealand since 1948. Unfortunately, the International Classification made no distinction by histological type for skin cancer, and about 6 years ago it was decided to abandon the collection of data about skin cancer altogether. The early figures, however, provide substantial basic information about the total incidence of skin cancer by age, sex, and locality. They will be referred to as "registered cases."

Since there was insufficient time to devise a forward study, it was necessary to collect data for this paper from existing records with the disadvantages of such retrospective inquiries. For this purpose all the in-patient, out-patient, and radiotherapy records at the public hospitals in Auckland, Wellington, and Christchurch were examined and all cases of skin cancer presenting for the first time from July 1, 1959, to June 30, 1960, were tabulated. Except for malignant melanoma, patients living outside these three urban areas were excluded. The records were in general very well kept and there is no reason to suppose that there were significant omissions in the data. These cases I have called "clinic cases." They were not, of course, comprehensive as some cases were treated privately and some not at all.

Some evidence on the latter point may be derived from figures which have been kindly supplied by Dr. Brian Hill, who is the sole dermatologist in the Hawkes Bay area. He has added cases treated by the sole radiotherapist. He belives these figures comprise practically all cases of skin cancer occurring in his well-defined locality. I refer to these cases as "Hawkes Bay cases."

In addition, for malignant melanoma, which is unfortunately so frequently a killing disease, we have a continuous record over many years from death certificates.

RESULTS

Malignant Melanoma

In Lancaster's work (*1*, *2*), in which he studied the geographical variation in mortality from melanoma, he concludes that sunlight may be a provocative factor. There is little doubt that deaths from malignant melanoma of the skin are a great deal higher in Australia and New Zealand than in Britain. It is mostly unlikely that this is due to artifacts in diagnosis and classification.

Fitzpatrick (*3*, *4*), demonstrated a tyrosine-tyrosinase reaction as characteristically present in malignant melanoma. He showed that this was not ordinarily present in junctional nevus tissue unless this tissue was stimulated by radiant energy of a wavelength found naturally in sunlight. When this happened such a tyrosine-tyrosinase reaction developed.

I do not know whether such a reaction can be measured in the pigment-bearing area of normal skin, whether there is a quantitative difference in the reaction between melanoma and irradiated junctional tissue, or what bearing this may have on malignant change in such tissue, but at least a prima facie case exists to incriminate sunlight as a suspect in the etiology of melanoma. Tables 3 through 6 compare data from cities in England and Wales, Australia, and New Zealand. I use these countries because we are kindred, come from common stock, and constitutional differences are minimized.

In Lancaster's (*1*) original paper he shows a difference in death rate in New Zealand for 1943–53 between the North and the South Islands. In the 10-year period of my current survey, 1950–59, this difference is no longer apparent. But then, there really is very little difference in practice between North and South Islands so far as sunlight and exposure to it are concerned, as I mentioned earlier. The sunniest spots are in the South Island, which probably compensates for the difference in latitude. For this reason and because of the smallness of our population, I have lumped data for both islands together and looked at the problem from another viewpoint—that of site.

I have divided the melanoma cases into two groups: (*a*) exposed sites, *i.e.*, head, neck, and hand only; and (*b*) all other sites.

The two groups contrast quite strikingly, as may be seen in table 1 and text-figure 1.

TABLE 1.—Death rates per million from malignant melanoma: exposed and other sites. New Zealand 1950–1959

	Male		Female	
Age	Exposed sites	Other sites	Exposed sites	Other sites
15–	1	3	—	—
25–	3	15	2	9
35–	6	19	1	20
45–	8	23	6	30
55–	13	28	6	16
65–	10	35	9	15
75+	31	19	34	36

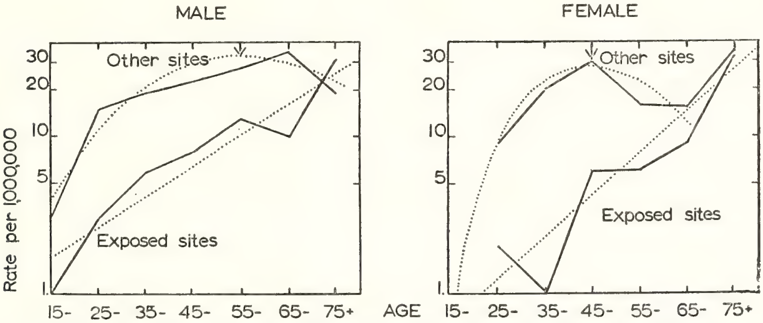
The exposed sites in both sexes show a steady increase with age and give an exponential type of curve. All other sites taken together, which clearly include a proportion of cases, more or less exposed, show a humping in middle age. The hump has a modal point some 10 to 15 years earlier in women than in men.

A possible explanation of this is that exposure, presumably to sunlight as in other forms of skin cancer, is a factor in inducing malignant change, is cumulative, and takes a relatively long time to act. Also, basically another factor(s), which may be hormonal, stimulates malignancy somewhat more explosively in the middle years.

There were 62 clinic cases of melanoma. In almost every case the nature of the tumor and its malignancy were confirmed by histology so that the vexing question, Have we included pigmented basal-cell carcinomas? did not arise.

Of the 62 clinic cases, 16 were of exposed sites. This is the same proportion, 85 to 334, that is found among the deaths, *viz*, about 25 percent. No figures exist in New Zealand on the site distribution of moles *per se* such as has been published by Pack, Lenson, and Gerber (*5*) in America, so we do not know the comparative blemish rate of the average New Zealand face. It is noteworthy that in the American series of Pack and his co-workers, of 1,235 lesions in 1,222 patients, about 33 percent of malignant melanomas were situated on the head, neck, and hand, *i.e.*, the exposed sites of my series.

This is greater than in New Zealand, even though the over-all prevalence of malignant melanoma is less than in New Zealand. Again, in London where the incidence of malignant melanoma seems substantially lower than in New Zealand, Harnett (*6*) in his monumental survey shows the high figure of 64 percent as occurring on exposed parts. Lancaster (*2*), in Australia, gives the figure of 32 percent of malignant melanoma occurring on the face alone, about the same as the American figure. Thus the higher incidence of malignant melanoma in Australia and New Zealand is not brought about exclusively by an excess of the condition in exposed sites, so we must look elsewhere than to sunlight to explain it fully. This does not mean that exposure to sunlight may not be one of a number of causes. The difference in age pattern between exposed and covered sites, already noted, gives some support to this view. It does suggest, however, that sunlight may not be as important a factor as has hitherto been postulated.



TEXT-FIGURE 1.—Malignant melanoma: exposed and other sites. Rates per million by sex.

It is estimated that the clinic cases represent approximately one half of the total yearly incidence of malignant melanoma in New Zealand, which therefore runs at about 120 cases each year. There was an average of 33 deaths over the last 10 years, which is only a quarter of the total incidence. I do not wish to discuss treatment, but would like to remark that this perhaps somewhat surprising difference between incidence and death rate represents a high 5-year survival rate, and this has been our experience in the past 20 years. We treat our cases almost entirely by surgery, and where radiotherapy is used it is often as a last resource or in a secondary role. This raises the questions: Are we diagnosing our cases earlier? Are our cases intrinsically less malignant?

Cancer of the Skin Other Than Malignant Melanoma

Here, so far as international comparison is concerned, we are on far more uncertain ground. In the absence of specifically planned and expensive surveys that could scarcely be justified, we are dependent for our data on a scrutiny of existing records, and however diligently we search there will always be a number of cases that will escape the net, and this proportion must vary greatly from place to place and country to country.

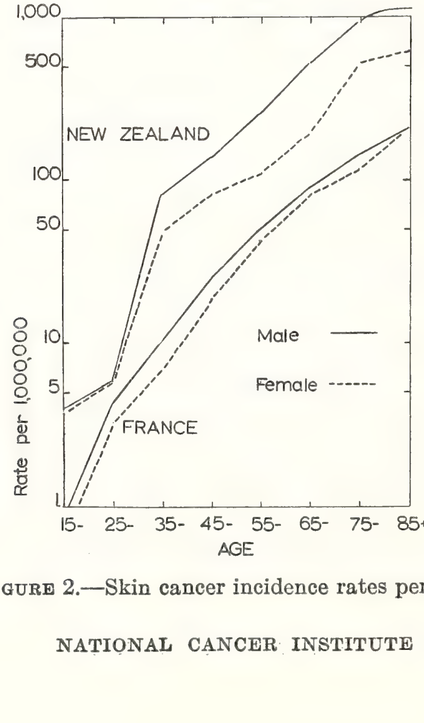
It is a widely held view in Australia, based on the assertions of a number of Australian writers, that skin cancer there is 10 times as common as in Britain. I do not wish to cast doubt on this statement which may well be true but it is not at all clear on what precise evidence the figure is based.

Incidence

Total.—The New Zealand incidence by age and sex of total skin cancer other than melanoma as shown by the clinic cases is set out graphically against similar figures calculated by Denoix (7) for France (text-fig. 2).

The New Zealand experience, unlike that of France, has a higher male: female sex ratio. Probably Denoix (7) understates the total French incidence, which would suggest that New Zealand males have 5 or 6 times the rate of French males and New Zealand females 2 or 3 times the rate of French females. The probable total yearly incidence in New Zealand is shown in table 2.

As no national figures exist for Britain, a comparison was made of the proportion of skin cases in Harnett's London Survey with the proportion of such cases in the New Zealand register from 1948 to 1955 (table 3). Both for males and females our figures are a little over 3 times as great



TEXT-FIGURE 2.—Skin cancer incidence rates per million.

as his. This fits in with other evidence and points to the conclusion that the incidence of skin cancer in New Zealand is about 3 times that in England. This would imply that the incidence in Australia is 3 times as high as in New Zealand. I am a little reluctant to believe this, though Belisario (*8*) gives figures for Australian cancer clinics that suggest an even higher rate in that country. It is far from clear, however, whether the Australian figures refer only to cases treated by radiotherapy or whether they include all cases in the area.

TABLE 2.—Probable total yearly incidence of skin cancer in New Zealand: rates per million

Malignant melanoma	55
Basal-cell carcinoma	1, 130
Squamous-cell carcinoma	380
Total	1, 565

TABLE 3.—Proportion of skin cancers to all other cancers

		Number of cases		Percentage of skin cases to all other cases of cancer		
		London (Harnett)	New Zealand		London (Harnett)	New Zealand
All sites, excluding skin cancer	Male	6, 376	13, 366			
	Female	6, 778	12, 460			
				Male	10. 5%	32. 8%
Skin cancer, excluding melanoma	Male	641	4, 383	Female	5. 7%	18. 1%
	Female	387	2, 260			
Comparable Australian figures (Belisario)	Brisbane					230%
	Sydney					150%
	Adelaide					100%

By types.—The different types of cancer included in the clinic series, most of which were verified by histology, are shown in table 4. As interest has been expressed in Bowen's disease and keratoacanthoma, figures for these conditions are also shown.

TABLE 4.—Relative incidence of skin cancers and precancers

Type of cancer	New Zealand (percent)	London (Harnett) (percent)	Sydney (Belisario) (percent)
Squamous-cell carcinoma	15	29	22
Basal-cell carcinoma	73	66	70
Malignant melanoma	7	4	Not included
Bowen's disease	2	1	1
Keratoacanthoma	3		7

A comparison of the proportions of basal-cell to squamous-cell carcinoma (table 5) in London, Australia, and New Zealand shows that squamous-cell carcinoma is relatively much more frequent in London.

The percentage of basal-cell carcinoma is higher in Australia than in Britain, but strangely it is higher in New Zealand than in Australia. The reason for this is not clear. When each sex is considered separately the proportionate distribution between them is the same in New Zealand as in London, so that one sex does not contribute more than the other to the difference. A possible explanation is that in Britain a substantial number of squamous-cell carcinomas are due to industrial and related causes. In Australia and New Zealand a much larger number are due to sunlight, and in Australia, where the sunlight is more intense, a squamous reaction once again becomes relatively as well as absolutely more frequent.

TABLE 5.—Proportions of basal-cell to squamous-cell carcinoma: New Zealand compared with London and Sydney

<table>
<tr><th rowspan="2">Type of carcinoma</th><th colspan="4">London (Harnett)</th><th colspan="2">Sydney (Belisario)</th><th colspan="4">New Zealand</th></tr>
<tr><th colspan="2">Number</th><th colspan="2">Percent</th><th>Number</th><th>Percent</th><th colspan="2">Number</th><th colspan="2">Percent</th></tr>
<tr><td>Both sexes</td><td colspan="2"></td><td colspan="2"></td><td></td><td></td><td colspan="2"></td><td colspan="2"></td></tr>
<tr><td>Basal-cell</td><td colspan="2">916</td><td colspan="2">70</td><td>2,466</td><td>76</td><td colspan="2">680</td><td colspan="2">83</td></tr>
<tr><td>Squamous-cell</td><td colspan="2">309</td><td colspan="2">30</td><td>792</td><td>24</td><td colspan="2">138</td><td colspan="2">17</td></tr>
<tr><td></td><td colspan="2">Male</td><td colspan="2">Female</td><td colspan="2"></td><td colspan="2">Male*</td><td colspan="2">Female*</td></tr>
<tr><td>By sex</td><td>Number</td><td>Percent</td><td>Number</td><td>Percent</td><td colspan="2"></td><td>Number</td><td>Percent</td><td>Number</td><td>Percent</td></tr>
<tr><td>Basal-cell</td><td>434</td><td>68</td><td>282</td><td>73</td><td colspan="2" rowspan="2">Not given by sex</td><td>369</td><td>83</td><td>228</td><td>87</td></tr>
<tr><td>Squamous-cell</td><td>207</td><td>32</td><td>102</td><td>27</td><td>77</td><td>17</td><td>34</td><td>13</td></tr>
</table>

*Excludes Hawkes Bay cases.

Etiology

There were only two skin cancers reported in Maoris, a small fraction compared to expected European rates. One was presumably of industrial origin (mineral oil) and the other followed a chronically discharging sinus. Only one other occupational cancer was discovered despite careful sifting of the data, and this contrasts markedly with Harnett's London series (*6*) in which occupational factors were relatively frequent, especially where industrial oil and tar were found. There was a small proportion of cases that followed trauma, usually of a trivial nature, and these cases, as Belisario (*9*) has pointed out, were probably in skin already affected by prolonged exposure to sunlight. A case in point, reminiscent of an old nursery rhyme, occurred in a woman who was bitten on the nose by a magpie while she had been hanging out clothes 2½ years earlier; the bite had never healed but developed into a basal-cell carcinoma. These data are given in table 6.

There were only two cases of penile cancer—a very low incidence—and probably due to the widespread custom of circumcision practiced in this country.

Sunlight, especially so far as it affects the blue-eyed and the blonde, has been well incriminated as the villain. There are two points I should like to raise with regard to sunlight and distribution by sites.

TABLE 6.—Antecedent factors

Agent	London (Harnett)								New Zealand							
	Basal-cell carcinoma				Squamous-cell carcinoma				Basal-cell carcinoma				Squamous-cell carcinoma			
	Male		Female		Male		Female		Male		Female		Male		Female	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Mineral oil, tar	14	3	1	—	30	14	—	—	1	—	—	—	1			
Other chemicals	8	2	1	—	2	1	—	—	—	—	—	—	—	—	—	—
Chronic skin changes due to drugs taken internally	—	—	—	—	1	—	—	—	—	—	—	—	1	1	—	—
Previous burn or scald	6	1	9	3	4	2	1	1	—	—	—	—	1	1	—	—
Trauma	77	18	34	12	46	22	23	23	13	4	3	1	—	—	—	—
Chronic sepsis and skin irritation	No comparable figures								5	1	3	1	5	7	2	6
Total number of cases	434		282		207		102		369		228		77		34	

In basal-cell carcinoma 97 percent were situated on the head and neck in London (Harnett) (*6*), 93 percent in New Zealand, and 91 percent in Sydney (Belisario) (*10*); that is, the higher the over-all incidence the smaller the percentage of lesions situated on the head and neck and the larger the number that appear on other regions of the body. Only 1 percent appeared on the hand in New Zealand (these were on the dorsum) but none at all were found on the hand in London. In his series, Belisario does not record the number of lesions on sites other than head and neck. This suggests that head and neck are the sites of choice, but with increasing exposure to sunlight other sites more often become affected.

The second observation concerns the distribution of sites on the face. Mackie and McGovern (*11*) describe changes in the elastic tissue of the skin induced by sun and postulate that they are the forerunners of cancerous change. I am sure this is a factor of the greatest importance, but if it were the sole factor concerned, skin cancer would be maximal on those areas of the face that receive the most sunburn. This is not so. There is a concentration of cancer, out of proportion to the size of the area of skin involved, from the inner canthus down the side of the nose and including the contiguous skin of the cheek, which is in an area especially rich in sebaceous glands.

It has been postulated that when men migrated northward in Europe they developed a blonde skin, which made the best use of the meager sunlight to manufacture vitamin D.

Perhaps the sunlight, in addition to converting a substrate to vitamin D, acts upon a similar precursor in the skin and develops in it a carcinogenic activity. This would explain the higher incidence in areas of the skin where there is a concentration of fatty material.

There is a further postulate: Is it not possible that some of the high incidence of skin cancer in Australia and New Zealand is due to the very high intake of fat, especially animal fat, in the diet? A recent survey by Hunter and Wong (*12*) showed that New Zealand diets are exceptionally high in cholesterol content and the blood cholesterol levels in a representative collection of people were unduly high.

A further discovery, the significance of which is obscure, was a high incidence of chronic lymphatic leukemia among males with skin cancer. There were 6 cases among 446 of basal-cell and squamous-cell carcinoma. The expected number was one fifth of a case. No case was found among the women.

Is it related to diet?

Is sunlight also a factor in causing lymphatic leukemia?

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SKIN CANCER IN AUSTRALIA[1]

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SUMMARY

The common occurrence of skin cancer in Australia is discussed, and the mortality rate compared with that of other countries. Although the incidence rate is high, the actual death rate is still low, but rises alarmingly in the older age groups. An assessment of the incidence rate in the State of Victoria disclosed that undetected skin cancer in that State occurs in about 1,300 per 100,000 population. It is possible that the situation is worse in most of the other States of the Commonwealth of Australia, and the reasons are discussed. An analysis is given of the histological types and age distribution of skin cancers in Western Australia during the years 1957–60. The high incidence of molluscum sebaceum (keratoacanthoma) in Australia is reported and discussed. In Australia about 1½ times as many individuals die of malignant melanoblastoma as of all other forms of skin cancer combined. The Western Australian material for the years 1957–60 was analyzed and showed a discrepancy between the sex ratios in the biopsy material and the death figures. The important etiological factors for the development of skin cancer and malignant melanoblastoma in the Caucasian population of Australia are discussed. Sunlight seems to play the major part as a carcinogenic factor for the inducement of skin cancers. This is particularly important in Australia as a sizable section of the population has a fair skin, being of Irish or Scottish descent. The action of strong sunlight as a carcinogenic factor in the development of malignant melanoblastoma as postulated by Lancaster and Nelson is discussed. The changing population structure of Australia due to postwar large-scale immigration will require a reassessment of the problem in 5 to 10 years.—NCI Monogr. No. 10: 153–156, 1963.

MALIGNANCIES OF the skin for various reasons are classified as "malignant melanoblastomas" (International Classification rubric 190) and "other malignancies of the skin" (International Classification rubric 191). The latter group mainly comprises squamous-cell carcinoma

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

[2] Most of the figures are based on information provided by the Western Australian Office of the Commonwealth Bureau of Census and Statistics, to which I feel much indebted. I am grateful to Dr. R. Kelsall, of the Department of Pathology, Royal Perth Hospital, for collecting the hospital cases, and Mr. H. Upenieks, Department of Medical Illustration, Medical School, University of Western Australia, for the preparation of tables, graphs, and photographs.

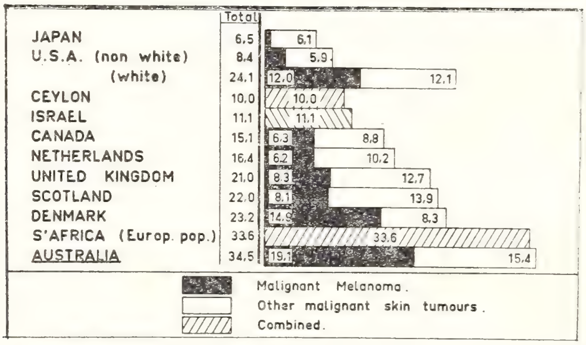
(epidermoid carcinoma), basal-cell carcinoma (rodent ulcer), plus a small group of rare cancers like mycosis fungoides, sweat-gland carcinoma, etc. In this article, for the sake of convenience, the words "skin cancer" are used for this latter group, except where differently stated.

FREQUENCY

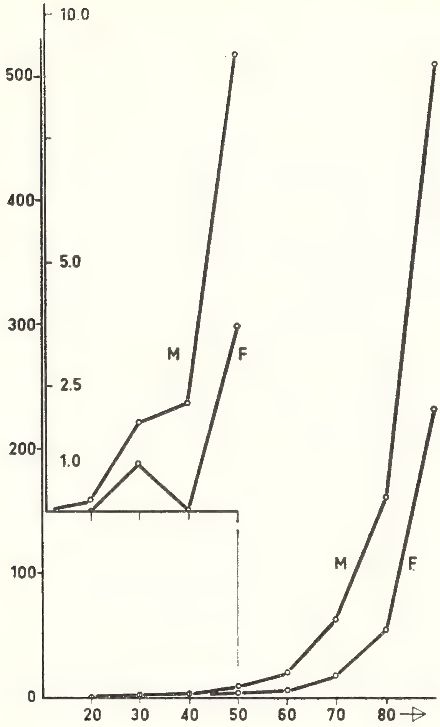
Skin Cancer

Skin cancer is considered to comprise more than 50 percent of all cancers in Australia (*1*). It has been estimated that it occurs at least 10 times as frequently as in Great Britain (*2*, *3*) and forms nearly one third of all skin conditions seen clinically (*1*). The clinicians, and to a certain extent the public, are aware of this high incidence. As a result of this fact and the high curability rate of most skin cancers, with the exception of malignant melanoblastoma, the mortality rates, although higher than anywhere else, are not alarming (text-fig. 1). In comparison with the mortality rate of all cancers combined in Australia, the death rate from skin cancer is definitely low. This cannot be a matter of complacency as the actual number of deaths still comprises about 140 a year, while it should be possible to reduce this figure to a few dozen of the comparatively rare anaplastic squamous-cell carcinomas and other more malignant conditions like mycosis fungoides. In addition, the death rate rises alarmingly in the older age groups (text-fig. 2). With an over-all increase in life expectancy, skin cancer might, over the years, become more important as a cause of death than is true at present. Death is usually the result of a rapidly growing and spreading anaplastic squamous-cell carcinoma (fig. 1). However, in Australia at least, every year a few patients die of a relentlessly extending and uncontrollable basal-cell carcinoma, which ultimately penetrates the skull (figs. 2 and 3).

Although the common occurrence of skin cancer in Australia has been appreciated for some time, very little is known about the actual incidence



TEXT-FIGURE 1.—Comparison of crude death rates in Australia from malignant melanoma (rubric 190) and other malignancies of the skin (rubric 191) with those of some other countries.



TEXT-FIGURE 2.—Skin cancer (rubric 191). Age-corrected death rates, Australia.

rate. To assess this incidence, the Department of Health of the State of Victoria made a recent survey of a statistical sample of the Victorian community (*4*). From these data it is estimated that in Victoria about 100,000 persons have treated or untreated skin cancer. A surprisingly high number of untreated and unsuspected skin cancers was found and roughly for every 4 treated about 3 untreated cases were detected. In a population of about 2.8 million the incidence is therefore extremely high. Yet, only 20 people died of skin cancer in Victoria in the year of this survey (1958). The figures do not allow a real assessment of the incidence rate as they do not include or exclude all treated cases. If we ignore these, the incidence of untreated cases is still 1,300 per 100,000 population in Victoria. It is likely that in most other States of the Commonwealth of Australia the incidence will be the same or higher, as all, with the exception of Tasmania, enjoy even more sun-hours than Victoria (table 1).

In Western Australia, with a population of roughly 720,000, about 1,000 cases of skin cancer are treated per annum by radiotherapy alone, while a large number is treated by surgical excision. As unfortunately not all of these are histologically confirmed, the total number so removed is impossible to assess. In the Royal Perth Hospital, the main teaching hospital of Western Australia, every year a large number of patients with skin cancer are treated radiologically or by surgery. In addition, a certain amount of other material from outside the hospital is received for histological investigation. The total number of patients so treated or investigated is about 400 per annum. The anatomical distribution of the 1,683 lesions observed in 1,667 patients in the 4 years 1957–60 is presented in table 2, while table 3 gives the distribution in age groups. The exposed parts of the body, in particular the skin of head and neck, are by far the most affected, as is common among the more lightly pigmented races. There were 16 multiple cancers at the time of treatment, and a clinically undiagnosed skin cancer unrelated to the cause of death was found 7 times at necropsy. It was impossible to assess how many patients had multiple lesions occurring at different times.

TABLE 1.—Distribution of sun-hours per annum over the capital cities of Australia

City		Hours
Hobart	(Tasmania)	2127. 3
Melbourne	(Victoria)	2222. 3
Canberra	(Austr. Cap. Territ.)	2426. 0
Sydney	(New South Wales)	2478. 6
Adelaide	(South Austr.)	2535. 2
Brisbane	(Queensland)	2713. 2
Perth	(West Austr.)	2835. 5
Average		2476

In our material basal-cell carcinoma is by far the most common (tables 2 and 3). It is doubtful if the figures for those treated by radiotherapy alone are truly representative as they are based on clinical diagnoses only. In the assessment of biopsies it is a common experience that the clinical diagnosis of basal-cell carcinoma has to be corrected to one of squamous-cell carcinoma. The radiotherapy figures are therefore not completely reliable as to types. The frequency of basal-cell carcinoma is high but the ratio of squamous-cell carcinoma to basal-cell carcinoma is considerably less than could be expected from the assessment of such ratios from elsewhere in the world. Both forms of skin cancer increase rapidly with age.

Molluscum Sebaceum (Keratoacanthoma)

This interesting, rapidly developing, and usually spontaneously healing lesion has received much attention in the last years, although it has been known for a considerably longer time. It is particularly the articles of MacCormack and Scarff (*5*) and Rook and Whimster (*6*) which seem to have aroused the interest of dermatologists throughout the world. Its relation to skin cancer is still doubtful. The condition is extremely common in Australia and our material contains 173 histologically confirmed cases (tables 4 and 5). This represents about 1 case to every 2 of squamous-cell carcinoma, a ratio even higher than reported by Belisario (*1*) who found a ratio of 1 to 3.

Table 2.—Basal-cell and squamous-cell carcinoma—Royal Perth Hospital 1957–60. Anatomical distribution and sex

	Histologically verified						Clinical diagnosis only: Treated by radiotherapy							
	Basal-cell carcinoma			Squamous-cell carcinoma			Basal-cell carcinoma			Squamous-cell carcinoma				
Localization	M	F	U	M	F	U	M	F	U	M	F	U	Total	Percent
Head and neck	217	112	1	186	53	1	367	308	—	94	66	—	1405	93. 23
Upper limb	40	14	—	48	33	—	3	7	—	11	8	—	164	
Trunk	22	13	—	19	10	—	2	—	—	2	1	—	69	4. 10
Lower limb	2	6	—	9	7	—	—	—	—	2	2	—	28	1. 66
Unknown	7	3	—	4	1	—	—	—	—	2	—	—	17	1. 01
Total	288	148	1	266	104	1	372	315	0	111	77	0	1683	100. 0
Combined	808						875							

TABLE 3.—Basal-cell and squamous-cell carcinoma—Royal Perth Hospital 1957–60. Age groups and sex

Age groups	Histologically verified						Total	Clinical diagnosis only: Treated by radiotherapy						Total	Combined totals		
	Basal-cell carcinoma			Squamous-cell carcinoma				Basal-cell carcinoma			Squamous-cell carcinoma						
	M	F	U	M	F	U		M	F	U	M	F	U		M	F	U
0–29	7	2	—	2	8	—	19	2	2	—	—	2	—	6	11	14	—
30–39	10	2	—	12	3	—	27	9	5	—	1	—	—	15	32	10	—
40–49	29	16	—	30	10	—	85	26	11	—	9	2	—	48	94	39	—
50–59	49	22	—	49	15	—	135	49	43	—	16	8	—	116	163	88	—
60–69	78	44	—	61	28	—	211	111	104	—	28	21	—	264	278	197	—
70→	103	56	—	103	37	—	299	175	144	—	54	39	—	412	435	276	—
Unknown	8	5	1	7	2	1	24	—	3	0	1	2	0	6	16	12	2
Totals	284	147	1	264	103	1	800	372	312	0	109	74	0	867	1029	636	2
Combined	432			368				684			183				1667		

TABLE 4.—Molluscum sebaceum—Royal Perth Hospital 1957–60. Anatomical distribution and sex

Localization	Male	Female	Unknown	Total	Percent
Head and neck	51	18	—	69	39.89
Upper limb	56	25	—	81	46.82
Trunk	5	3	—	8	4.62
Lower limb	6	2	—	8	4.62
Unknown	2	5	—	7	4.05
Total	120	53	0	173	100

TABLE 5.—Molluscum sebaceum—Royal Perth Hospital 1957–60. Age groups and sex

Age groups	Male	Female	Unknown	Total
0–29	7	—	—	7
30–39	7	1	—	8
40–49	12	7	—	19
50–59	24	10	—	34
60–69	29	10	—	39
70→	38	18	—	56
Unknown	3	7	—	10
Total	120	53	0	173

Malignant Melanoblastoma

In the years 1956–60 at least 1,032 people died in Australia from this malignancy as against 678 for all other malignancies of the skin. The age-corrected death rate per million over these years is given in text-figure 3 and clearly indicates an increase with age. The sex ratio is 1.35. In the same years 42 males and 31 females died in Western Australia of malignant melanoblastoma, showing an identical sex ratio of 1.35.

In the 4 years 1957–60 malignant melanoblastoma was diagnosed at the Royal Perth Hospital in 99 patients (confirmed by histology in 95), 39 men and 58 women; in 2 biopsies received from elsewhere the sex was not recorded. The sex ratio is only 0.68. The difference in sex ratios seen in mortality and biopsy figures can be explained in various ways. Women seek earlier treatment for what is still a small blemish of the skin than men in general do. Thirty-three males and 28 females died of malignant melanoblastoma in these 4 years in Western Australia. The high mortality of malignant melanoblastoma is illustrated in the relatively small margin between the number diagnosed and the number of people who died of the condition in the same years. This compares most unfavorably with the relevant figures for the same period in regard to other malignancies of the skin. In 1957–60 only 12 males and 6 females died of other malignancies of the skin, while in that time basal-cell and squamous-cell carcinoma were diagnosed at the Royal Perth Hospital in

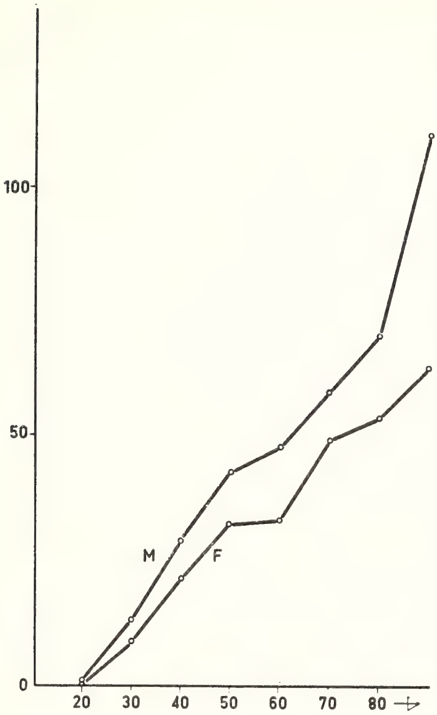
1,667 patients. The anatomical distribution and age grouping of the 99 cases of malignant melanoblastoma are shown in table 6.

Table 6.—Malignant melanoblastoma—Royal Perth Hospital 1957–60. Age groups, anatomical distribution, and sex

Age groups	Male	Female	Unknown	Total
0–29	2	7	—	9
30–39	10	11	—	21
40–49	8	13	—	21
50–59	7	5	—	12
60–69	4	9	—	13
70→	6	11	—	17
Unknown	2	2	2	6
Total	39	58	2	99
Localization				
Head and neck	11	11	—	22
Upper limb	6	9	—	15
Trunk	13	9	—	22
Lower limb	6	25	—	31
Secondary prim. unknown	2	1	1	4
Unknown	1	3	1	5
Total	39	58	2	99

Precancerous Conditions and Other Forms of Malignancy of the Skin

Premalignant keratosis, Bowen's disease, and some rare malignancies of the skin were diagnosed in 151 cases; 114 were solar keratosis (including cutaneous horn) with a preponderance in males and increasing with age. The age distribution of the 17 diagnosed as Bowen's disease suggests that, in fact, a number of solar keratoses with Bowen-like appearance have been incorporated in this figure. Other primary malignancies of the skin include: 5 cases of mycosis fungoides, 3 anaplastic unclassifiable carcinoma, 3 trichoepithelioma, 2 adenoid cystic carcinoma, 2 sebaceous carcinoma, 3 clear-cell hydradenoma, and 2 dermatofibrosarcoma. Clear-cell hydradenoma (epithelioma) is included because some of those reported in the literature behaved in a malignant fashion.



Text-figure 3.—Malignant melanoblastoma (rubric 190). Age-corrected death rates per million population, Australia 1956–60.

ETIOLOGY

Much has been written about various factors influencing the development of skin cancer and malignant melanoblastoma. It is not the purpose of this paper to reiterate these factors and I will limit myself to those which seem to be of particular importance for the Australian population.

Skin Cancer

The most important and outstanding single factor which influences the high incidence rate of skin cancer in Australia is most likely the ultraviolet radiation in the sunlight. The Australian climate is dry and sunny, with considerably more sun-hours than are experienced in most other countries where white people live (table 2). The average compares extremely favorably with a total of about 1,500 sun-hours in cities like London and Amsterdam. Even the lowest number of sun-hours in Australia (Hobart, Tasmania) is nearly 1½ times that of those cities. In addition, the day temperature during at least 9 months of the year is usually sufficiently high to induce people to outdoor activities. The clothing is light. Swimming, surfing, and fishing along the coast are favorite pastimes. Tennis is a winter sport! The actual number of sun-hours, therefore, in itself does not express the amount of exposure to sunlight. Neither does it express the amount of ultraviolet radiation in the different parts of Australia. Brisbane, Queensland (lat 27°30 south), enjoys fewer sun-hours than Perth, Western Australia (lat 31°58 south), but ultraviolet radiation increases toward the equator. It may well be that the actual exposure to ultraviolet of the population in Queensland is higher than in the southern half of Western Australia where the majority of Western Australia's population lives. Another important factor is the skin pigmentation and skin texture of the individuals at risk. A large section of the population of Australia is of British descent and, in fact, Irish or Scottish in origin. Among these latter groups, lack of skin pigmentation is extremely common. Even those Irish individuals with dark and even black hair commonly have blue eyes and a particularly pale skin which burns easily. Molesworth (*2*) many years ago drew attention to these facts.

It is difficult to assess the extent to which the dryness of the climate alters and predisposes the skin. Experiences from the tropics (*7*, *8*) seem to indicate that this factor is of lesser importance than the amount of ultraviolet radiation.

Molluscum Sebaceum

The extremely common occurrence of this condition in Australia, the anatomical distribution (table 5), and the age grouping (table 6) suggest that some common etiological factor is involved with squamous-cell carcinoma, as has been suggested by others.

Malignant Melanoblastoma

Little is known about the etiological factors influencing the development of malignant melanoblastoma. It is generally accepted that this malignancy, in an overwhelming majority of cases, arises from a preexistent pigmented nevus, lentigo or ephelide, although it is held that development in a previously unblemished skin is possible. Hormonal influences seem to play a part, as malignant melanoblastoma before puberty is extremely rare, and, in addition, pigmented nevi commonly develop at puberty, sometimes in large numbers. It is probable that repeated trauma may influence the development of this kind of malignancy. Its frequent occurrence on the sole of the foot in native populations supports this view (*9*, *10*). Lancaster has suggested (*11*, *12*) that exposure to sunlight is an important factor. His statistical data of frequency rates in relation to geographical latitude are impressive. A clinical comparison, by him and Nelson (*12*), of hair color, skin color and texture, color of eyes, and reaction to sunlight, of melanoma patients with those with other forms of skin cancer and patients with cancers of another primary origin, all of the same age group and sex, strongly supports his view. The anatomical distribution of the lesions, however, does not support this theory.

It is possible that different and unrelated factors can independently influence the causation of this form of malignancy. It is generally accepted that squamous-cell carcinoma of the skin may not only develop in old scars and chronic ulcerations but may be induced by various forms of radiation and chemicals. There seems to be no reason, therefore, why in malignant melanoblastoma a common etiological factor should be present. It has to be realized that the reactive possibilities of the cells, and therefore the whole body, are limited. A particularly characteristic pathological lesion may result from a variety of causes. On the evidence presented by Lancaster and Nelson (*12*), excessive exposure of a sensitive skin to sunlight seems to be one of those etiological factors in a number of malignant melanoblastomas. How important has still to be assessed. It is unlikely that sunlight plays a major part in all cases or even the majority.

CONCLUSION

It seems that the warm, dry, and sunny climate of Australia is responsible for the exceedingly high incidence of skin cancer in its white population of mainly British descent. In particular, those of Scottish and Irish descent are prone to be afflicted. Malignant melanoblastoma most likely also develops under the influence of excessive exposure to sunlight of patients with a particularly sensitive skin.

As the composition of Australia's population is rapidly changing due to immigration from other European countries, a reassessment of the situation will be of value in future years. Such an assessment will have to take into account the country of origin, time and place of residence in Australia, age group, color of hair and eyes, texture of the skin, occupation of the individuals, and outdoor activities. Particularly in relation to malignant melanoblastoma, further investigations along these lines and those previously followed by Lancaster and Nelson (*12*) should be made, both in Australia and elsewhere.

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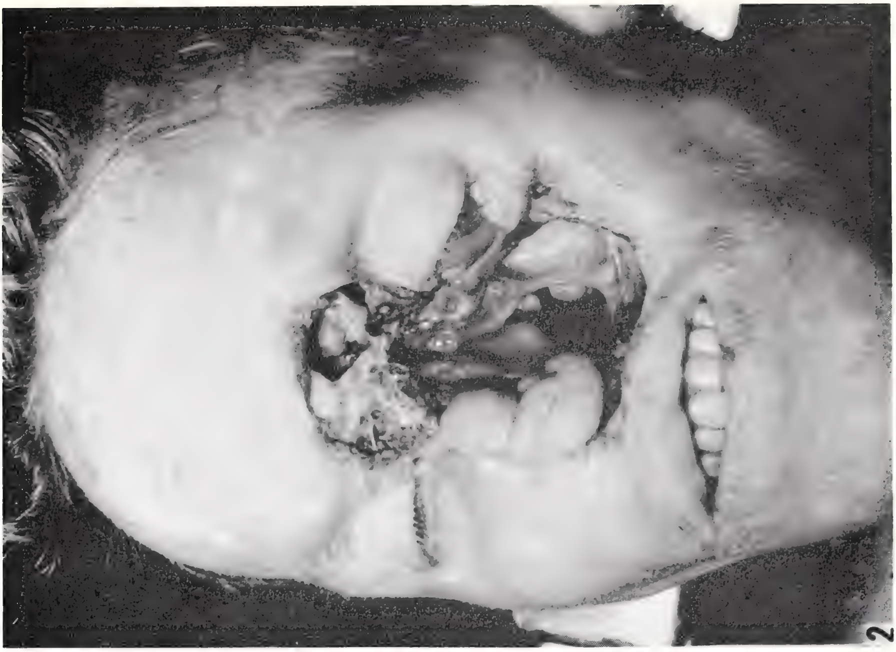
Plate 11

Figure 1.—Anaplastic squamous-cell carcinoma of scalp in male aged 80. Death followed rapidly after penetration of the skull.

Figure 2.—Incurable basal-cell carcinoma of many years' duration, with extensive destruction of nose and left cheek.

Plate 12

Figure 3.—Extension of basal-cell carcinoma along the leptomeninges. Hematoxylin and eosin. × 40



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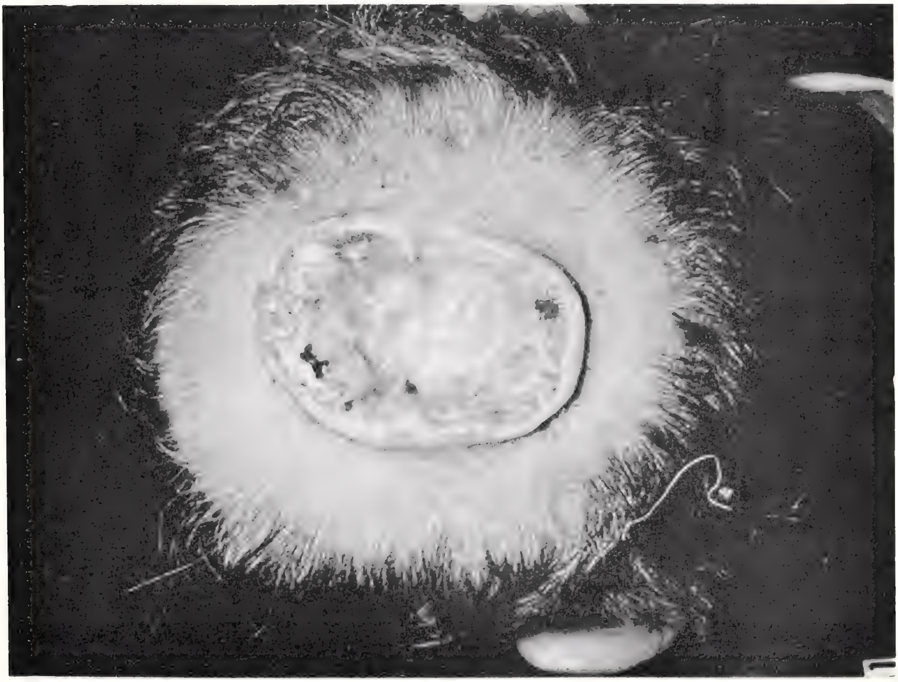

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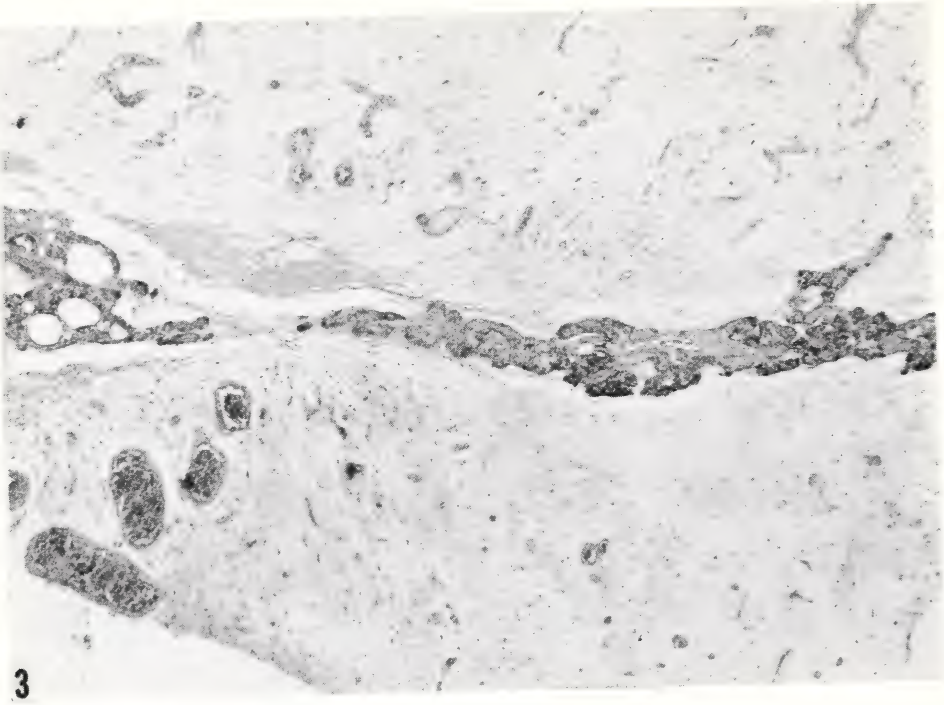

3

TEN SELDAM

SKIN CANCER IN NEW GUINEA NATIVE PEOPLES[1, 2]

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SUMMARY

An introduction to the country and population of New Guinea is given, with particular reference to the Australian (eastern) half. Ninety-nine skin cancers, including 4 cases of malignant melanoblastoma, were diagnosed for 1958–July, 1961, in natives of New Guinea. These tumors are discussed in regard to type and anatomical distribution. The preponderance of squamous-cell carcinoma on the leg is striking, and the etiological factors leading to this particular localization (tropical ulcer, trauma, yaws, burns) are discussed. The lack of complete protection against the ultraviolet in sunlight by deep pigmentation of the skin is stressed in relation to the 4 observed basal-cell carcinomas. Trauma seems to play a major part in inducing the occurrence of malignant melanoma on the foot and leg. Some of the difficulties regarding diagnosis, both clinical and pathological, are mentioned.—NCI Monogr. No. 10: 167–179, 1963.

THE ISLAND of New Guinea is the second biggest in the world, only Greenland being larger. It is separated from Australia by the Torres Straits and is situated just south of the equator. The eastern half, which is under Australian administration, is divided into the Territories of Papua and New Guinea, which also include a string of islands mainly to the north and east of New Guinea straddling the equator. The area comprises about 160,000 square miles for the mainland and 23,000 square miles for the islands. The Territories are administratively subdivided into areas, which are again subdivided into districts. The native population is about 1.9 million (table 1). In addition, there are about 19,000

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[6] Some of the clinical photographs have been provided by Dr. F. G. Smyth, General Surgeon, Taurama General Hospital, Port Moresby. We are indebted to Mr. H. Upenieks, Department of Medical Illustration, Medical School, University of Western Australia, for the preparation of the tables and most of the photographic work.

Table 1.—Population of Australian New Guinea as divided over the different districts

PAPUA	Western District	51, 000	
	Gulf	67, 000	
	Central	89, 000	
	Milne Bay	85, 000	
	Northern	47, 000	
Total			339, 000
N. G. HIGHLANDS	Eastern	323, 000	
	Western	242, 000	
	Southern	137, 000	
Total			702, 000
N. G. NORTH COAST	Morobe	182, 000	
	Madang	123, 000	
	Sepik	235, 000	
Total			540, 000
PACIFIC ISLANDS	Manus	16, 000	
	N. Ireland	37, 000	
	N. Britain	102, 000	
	Brit. Solomon Islands	108, 000	
	Bougainville	51, 000	
Total			314, 000
Combined totals			1, 895, 000

Caucasian (mainly Australian) and 5,000 Asian nonindigenous persons living in the Territories.

The country is extremely rugged with mountains ranging to 15,000 feet and over, which enclose, at about 5,000 feet, a comparatively level central depression. The population is of mixed ethnic origin and is in general deeply pigmented. The economy is mainly a very primitive subsistent agricultural one, with fishing along the coast. A large section of the population still lives under extremely primitive conditions. This applies particularly to the half million natives in the highlands, who, due to the inaccessibility of the country, were discovered only 30 years ago.

New Guinea is probably the only place in this world where a considerable part of the population still lives under stone-age conditions, or did so until recently. The vegetable gardens are worked with fire-hardened wooden sticks; stone adzes and stone arrow and spear heads, although becoming rarer, are still in use in various parts, while the body covering is a bare minimum. The natives take with alacrity, however, to the commodities which Western civilization can offer (fig. 1). In a tropical climate, and under these conditions, infectious diseases like malaria, tuberculosis, and the dysenteries take a heavy toll. The Public Health Services, against this background, have to concentrate for the time being on the combat of these infectious diseases, although individual treatment is far from neglected.

Cancer occurs in only about 3 percent of hospital patients and from a Public Health point of view is not yet of great importance. From the scientific point of view this is different and cancer becomes of extreme importance, as Western sophistication has not been introduced to a large section of the population for sufficiently long to have altered significantly the carcinogenic influences which may play a part under primitive conditions. This is fully appreciated by the Public Health authorities, who, from the inception, have given their sympathy and full support to a Tumor Survey initiated by one of us (L.A.).

This paper deals with skin cancer as encountered among the native population of the Australian part of New Guinea for the period 1958 to mid-1961.

RESULTS

The total number of malignancies of the skin diagnosed in this period was 99, of which 67 were confirmed by histology: There were 5 cases of malignant melanoblastoma, 4 of which were localized on the sole of the foot and 1 on the leg; basal-cell carcinoma was present in 4 cases, the remaining 90 being squamous-cell carcinoma. A striking feature was the anatomical distribution of the lesions, which were mainly localized on the leg and foot (table 2). The growths were usually very extensive and many had already formed metastases when first seen. Although most of these secondary formations were in the regional lymph nodes, 1 patient had pulmonary metastases. Of particular interest were 2 albinos, 1 of whom had multiple skin cancers, including the trunk area (figs. 2 and 3). Although the actual frequency of albinism among the population is unknown, the condition is not extremely rare. Several show partial albinism (piebaldism). However, in the total incidence of skin malignancies among the native population, albinism plays only a minor role.

TABLE 2.—Skin cancer in Australian New Guinea 1959–July, 1961—anatomical distribution and type

	Squamous-cell carcinoma	Basal-cell carcinoma	Malignant melanoblastoma	Total
Head and neck	6	4	—	10
Upper limb	2	—	—	2
Trunk	10	—	—	10
Leg	57	—	1	58
Foot	14	—	4	18
Unknown	1	—	—	1
Total	90	4	5	99

Etiological Factors

Sunlight.—How far pigmentation of the skin will protect against the carcinogenic influence of the ultraviolet rays in sunlight depends on the amount of pigmentation, the amount of exposure, and the amount of ultraviolet during exposure. That pigmentation *per se* is no absolute protection has been observed (*1–3*). From our material it is interesting to note that the 4 basal-cell carcinomas, which were all localized on face and head, occurred in highland natives (fig. 4). In these latitudes and in higher altitudes the ultraviolet radiation is extremely strong. It seems likely, therefore, that the deep pigmentation of the skin of most of the natives of New Guinea forms a considerable but not complete protection. This becomes even more evident from the fact that in the same period 3 of the 4 observed basal-cell carcinomas were histologically confirmed in Perth, not less than 15 of such lesions were diagnosed from Caucasians living in the same areas. The total number of Caucasians in these areas is only about 10,000.

Yaws.—It has repeatedly been reported from other Asian countries that skin cancer develops occasionally in old scars and depigmentations resulting from previously experienced yaws (*4, 5*). Yaws used to occur frequently in New Guinea natives, but, as a result of an extensive campaign by the Public Health Department to eradicate the disease, it nowadays is rarely seen. Aftereffects of the disease, in the form of scars and malformations, are still commonly encountered, but no evidence was forthcoming that these had a major part, if any, in the cases of skin cancer here reported.

Traumatic scars, including burns.—A population going barefooted and living nearly naked in or on the border of tropical jungle, and who, until recently, were subject to regular tribal wars, will clearly be prone to repeated trauma. Such trauma, if not directly leading to death, may easily leave ugly scars because of ignorance of antisepsis and lack of adequate treatment.

The mountain and highland people try to protect their nakedness against the night cold by wood fires inside their huts, and it is not surprising that within the small confines of these humble dwellings accidents easily happen, with resulting burns.

A number of such cases in the scar of either a previous trauma or burn was observed (figs. 5 and 6).

Tropical ulcers.—Tropical ulcers on the leg are commonly seen. A large number have become chronic, sometimes of many years' duration, when the patient first requests treatment by European doctors. The general health of the natives is poor. Anemia due to malaria and hookworm infection is common. In addition, other diseases like the dysenteries and a poor, mainly vegetarian diet of low protein content contribute to a lowering of the resistance of the individual against the development of tropical ulcers, and, once occurring, the healing is retarded. Experiences in the prisoner of war camps in the Pacific area during the recent war confirm this opinion. In a number of these chronic ulcers, squamous-cell carcinoma develops after a long time (figs. 7 and 8). In 1955 Backhouse (*6*) reported a number of cases which were observed in Melanesians from New Britain, and Saave (*7*) made similar observations on that island. The clinical appearance is not always a guide as to the correct diagnosis. The alternating breakdown and repair in these wounds, which are gradually extending and encroaching on the surrounding tissue, lead to a pseudoepitheliomatous hyperplasia which can easily be mistaken for malignancy (fig. 9). Even an experienced histopathologist will repeatedly feel uncertain as to the correct nature of the lesion he is investigating. Involvement of the underlying bone has sometimes taken place without this necessarily indicating the presence of malignancy. Occasionally only one of numerous blocks taken around the edge of these lesions will ultimately reveal its true nature (figs. 10, 11, and 12). This possibility of squamous-cell carcinoma developing in a chronic tropical ulcer must not be underestimated, as is demonstrated in those patients where rapid recurrence takes place after extensive plastic surgery or where secondary formations are present. In our material one fifth of the patients had metastases when first seen.

DISCUSSION

The anatomical distribution of skin cancers in New Guinea natives follows a similar pattern to that observed in other Asian and African populations. The high proportion of skin cancers occurring on the lower limb has been reported before from such countries and in New Guinea is even higher than in the neighboring country of Indonesia, where about one third of the cases had this localization (*1, 3–5*). Incidence rates for the two countries are not available for comparison and it is thus not possible to decide if this difference is the result of better over-all medical care in Indonesia, better protection of the deeper pigmented skin of the New Guinea natives against sunlight, or both. It may well be that Indonesians will have a larger number of skin cancers in other sites of the body as a result of the much lighter pigmentation of their skin. If so, the percentage of skin cancers on the lower limb would then be relatively less.

Chronic trauma seems most likely to be mainly responsible for the localization on the lower limb of the 5 malignant melanoblastomas which were observed.

The number of cases is small, but may not present a true picture. This becomes more evident when it is realized that the number diagnosed by pathology in 1960 alone is more than the combined number of the previous years (43 in 1960; 38 in 1958–59). The number of native patients with this condition and seen by European doctors is likely to rise temporarily as confidence in Western medicine increases. After some time, as a result of preventive treatment and improved health, a gradual drop in number can be expected.

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PLATES

Plate 13

Figure 1.—Native from the Eastern Highlands with umbrella.

Figure 2.—Female albino with squamous-cell carcinoma of right supraclavicular and cervical region.

Plate 14

Figure 3.—Same female albino as shown in figure 2 with squamous-cell carcinoma over right scapula and multiple subcutaneous metastatic nodes in cervical and paravertebral region. This woman also had innumerable hyperkeratotic lesions of the face, trunk, and limbs.

Figure 4.—Woman from the Eastern Highlands with pigmented basal-cell carcinoma of nose.

Plate 15

Figure 5.—Squamous-cell carcinoma in old spear injury on anterior chest wall.

Figure 6.—Extensive squamous-cell carcinoma of left elbow developed in old scar of burns. Hand is malformed by previous yaws.

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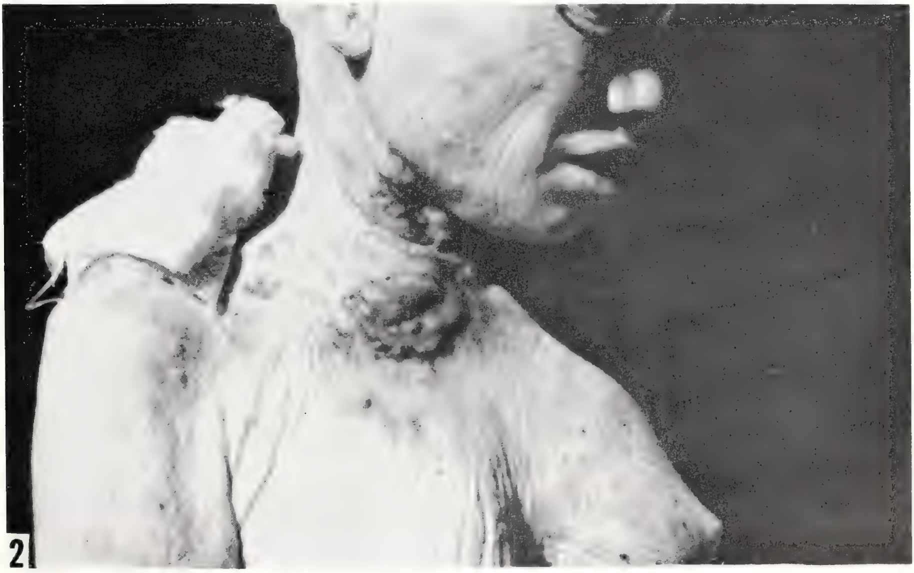
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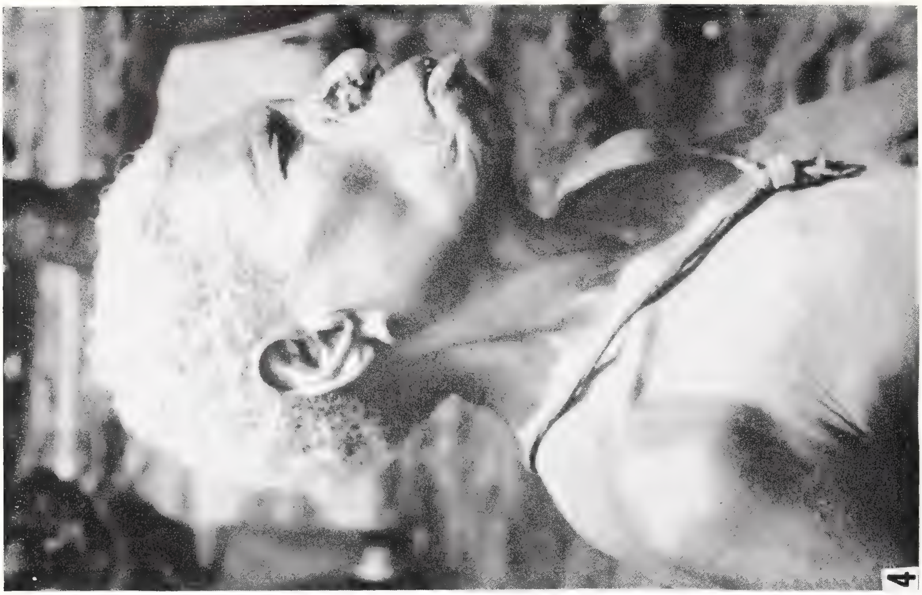

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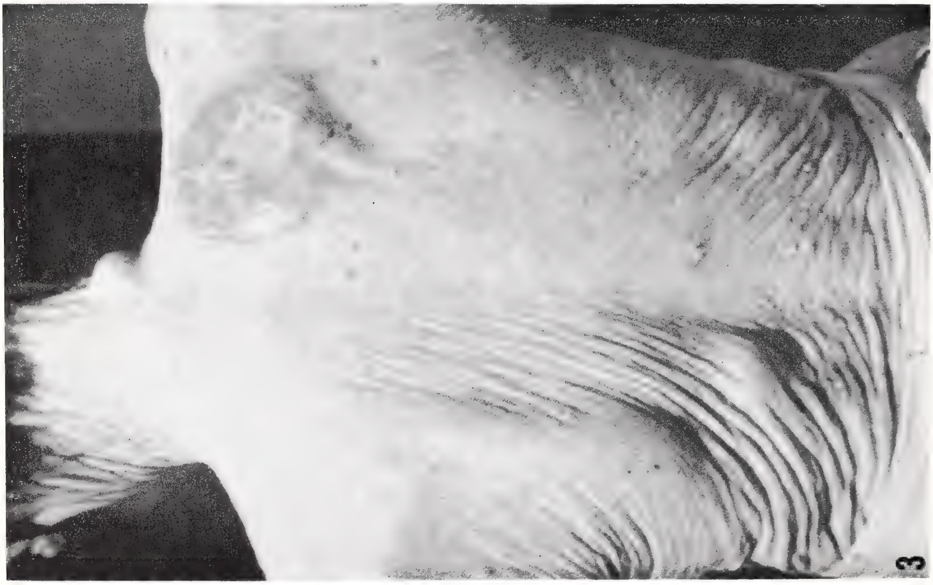

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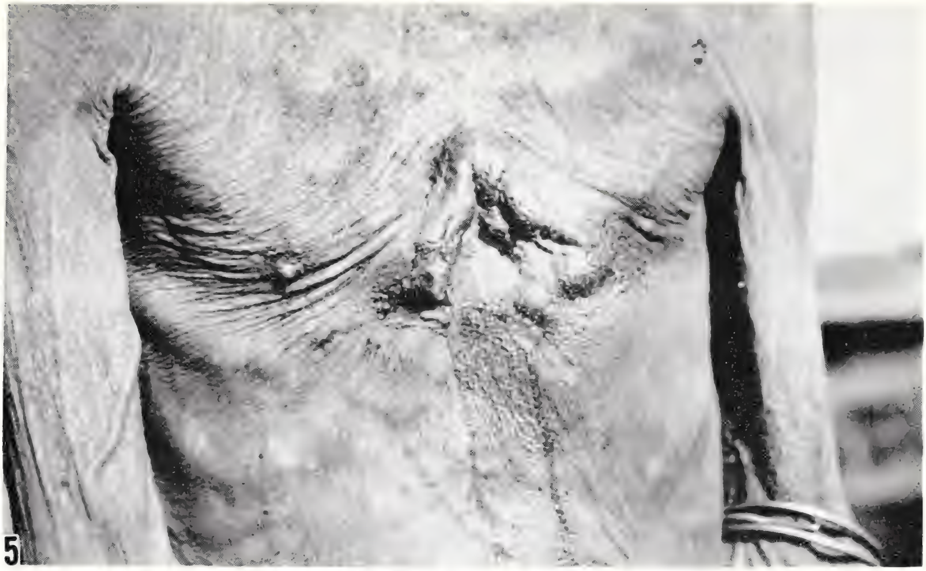
ATKINSON *et al.*



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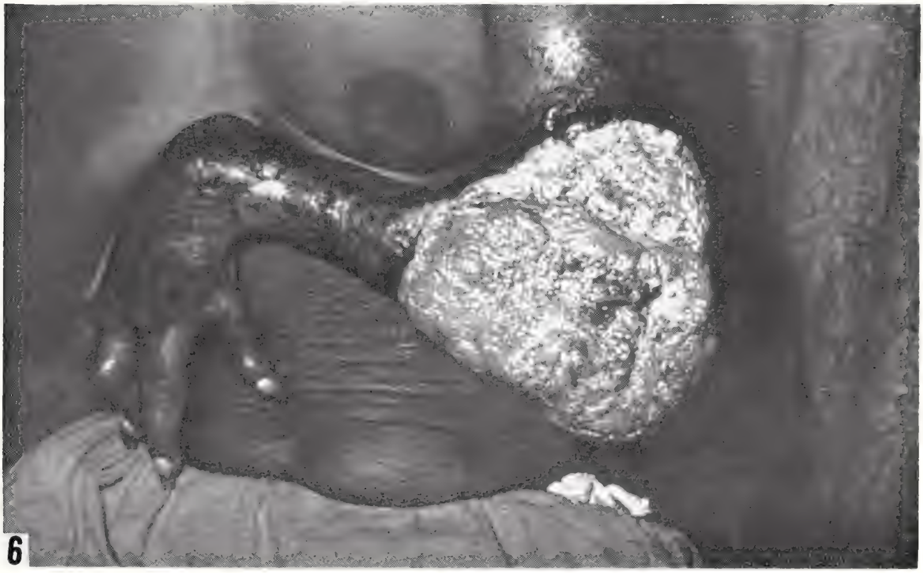

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PLATE 16

FIGURE 7.—Squamous-cell carcinoma which developed in chronic tropical ulcer on anterior aspect of leg.

FIGURE 8.—Squamous-cell carcinoma which developed in chronic tropical ulcer on anterior aspect of leg.

PLATE 17

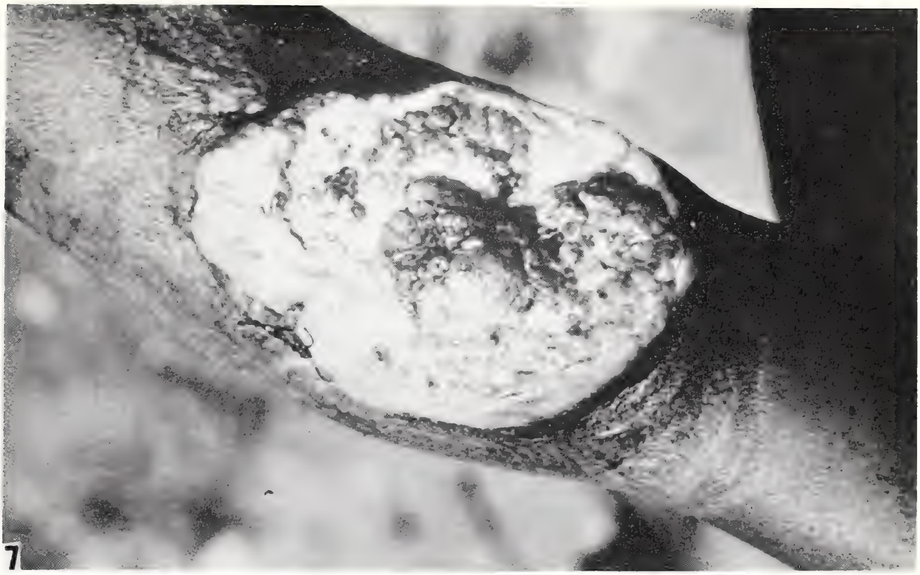
FIGURE 9.—Chronic tropical ulcer of anterior aspect of leg with fungating granulomatous growth in center. Clinically carcinoma, but histologically only chronic nonspecific inflammation with pseudoepitheliomatous hyperplasia.

FIGURE 10.—Pseudoepitheliomatous hyperplasia at edge of chronic ulcer on leg. Hematoxylin and eosin. × 5

PLATE 18

FIGURE 11.—Involvement of tibia in chronic ulceration of leg. Hematoxylin and eosin. × 16

FIGURE 12.—Squamous-cell carcinoma in chronic ulceration of leg. Hematoxylin and eosin. × 16



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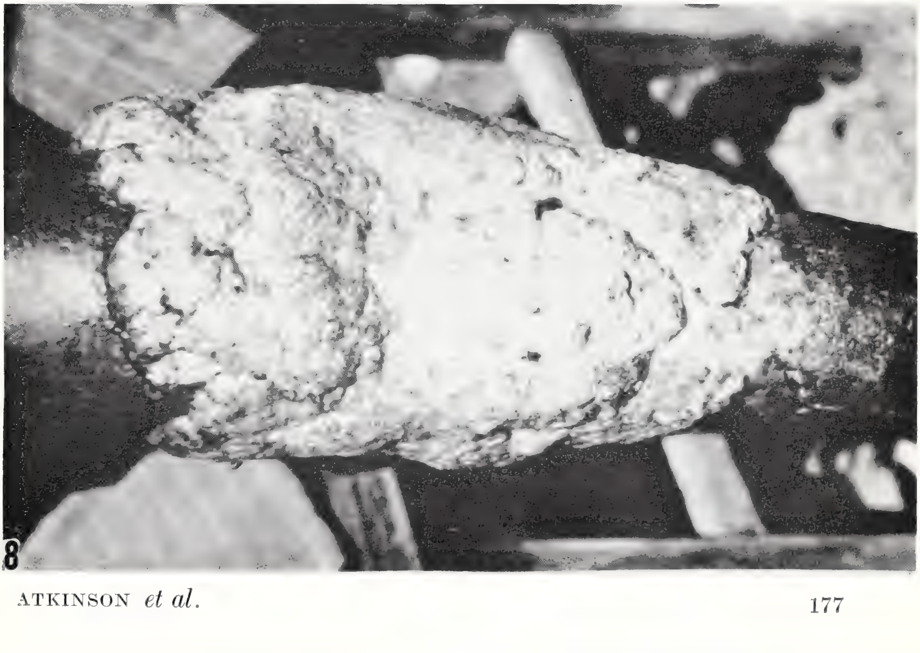

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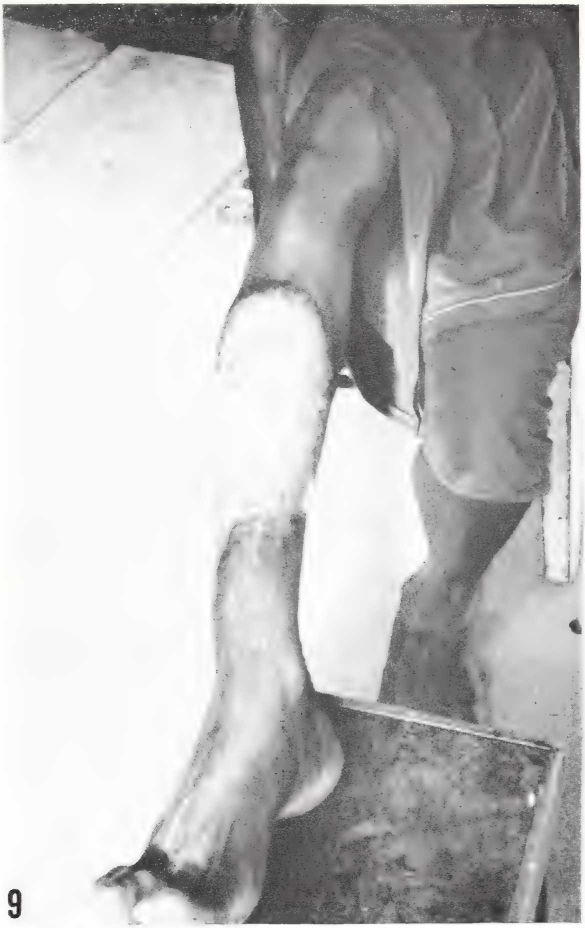

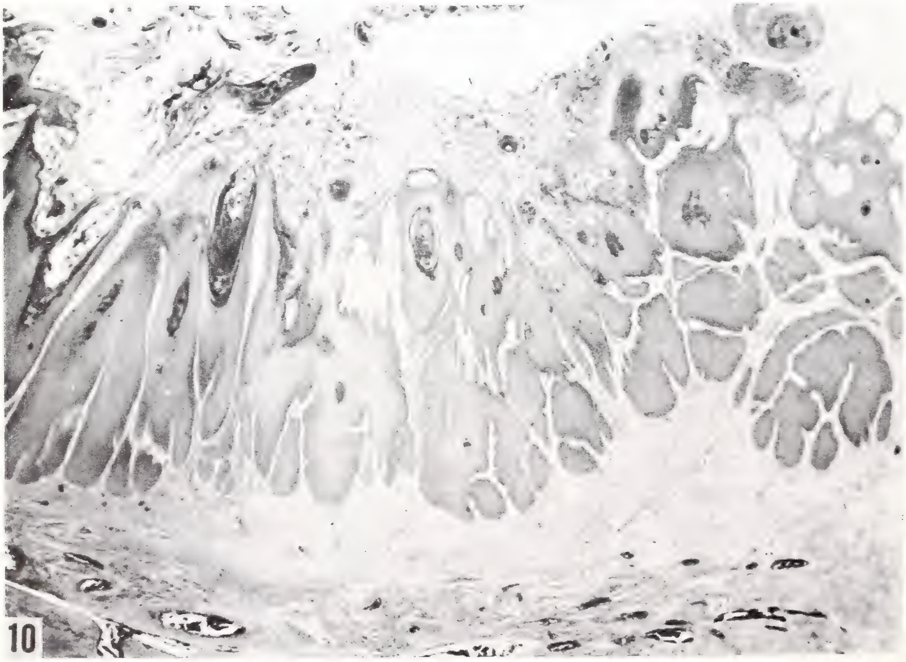

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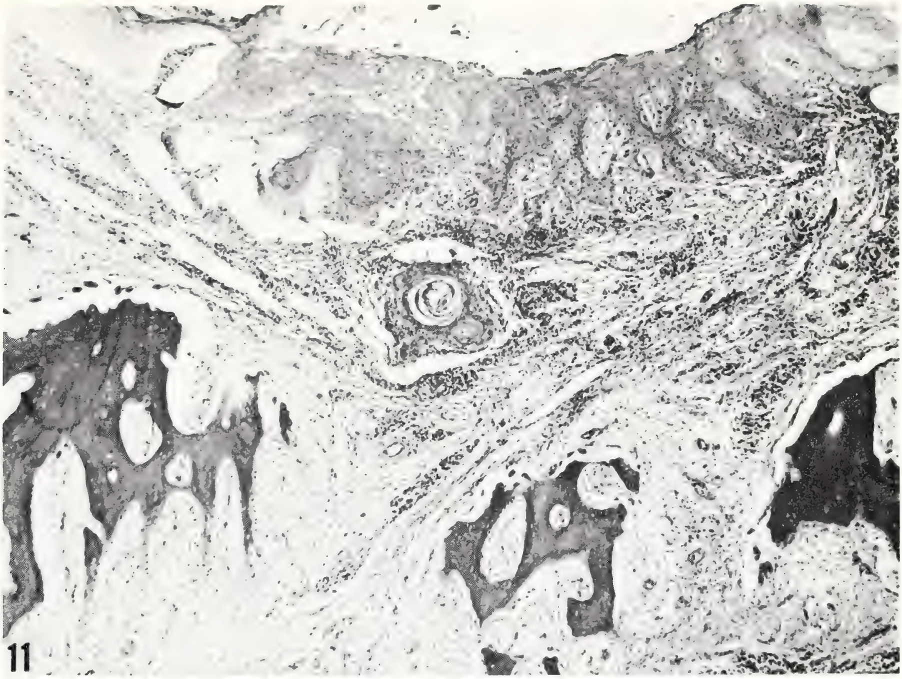

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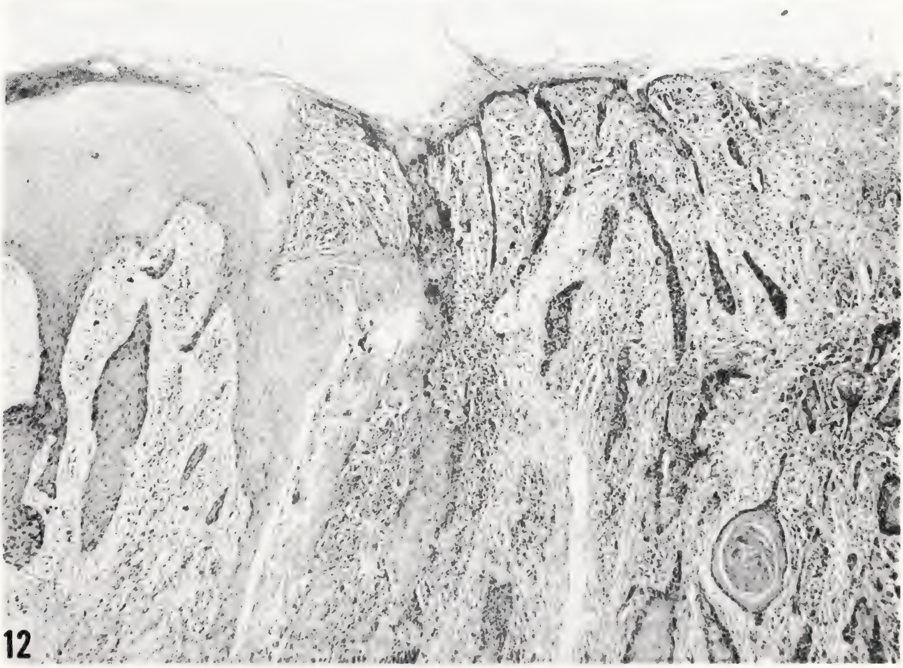

12

ETHNIC DIFFERENCES IN SKIN CANCER IN HAWAII[1]

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SUMMARY

Hawaii is a natural laboratory for the study of ethnic differences in skin cancer because it has a variety of racial groups living under the same climatic conditions. The major ethnic groups and their respective percentages of the population are Caucasians, 32 percent; Japanese, 32 percent; and Hawaiians and part-Hawaiians, 14 percent. The people are exposed to heavy year-round solar radiation, and the temperature range is from 65 to 85 degrees Fahrenheit. Studies conducted through hospital records and physicians' reports have shown a high skin cancer rate for Caucasians in Hawaii compared to non-Caucasian groups. The skin pigmentation of the non-Caucasian groups is probably the most important factor in the lower incidence. Caucasians in Hawaii apparently have a higher skin cancer rate than they have in selected areas of the mainland United States. This is probably due to exposure to a more carcinogenic type of solar radiation than is encountered in the mainland areas chosen for comparison. Sunshine is apparently more carcinogenic in lower latitude areas such as Hawaii than it is in higher latitudes. The absence of rest periods from exposure to ultraviolet radiation may also be responsible in part for the Caucasian rate in Hawaii being higher than it is in the mainland of the United States.—NCI Monogr. No. 10: 181–189, 1963.

ETHNIC DIFFERENCES in skin cancer in Hawaii have been of considerable interest to practicing physicians, especially dermatologists, and to public health workers for many years. This interest has been fostered at least partially by the well-known year-round exposure of the people to heavy solar radiation and to the heterogeneous ethnic composition of the population.

Approximately 32 percent of the residents are Caucasians and 32 percent are of Japanese extraction. About 14 percent are Hawaiians and part-Hawaiians, while 11 percent are Filipinos, 6 percent are Chinese, and 5 percent are in other ethnic groups, such as Koreans, Samoans, and Negroes.

In the discussion of ethnic differences, comparisons will usually be made between the 3 major groups, *i.e.*, Japanese, Caucasians, and Hawaiians and part-Hawaiians. However, in some instances for special reasons, other ethnic groups may be mentioned. Hawaii is considered a natural laboratory for the study of ethnic variations in skin cancer because of the many racial groups living under the same climatic conditions. Ethnic differences in several types of cancer have already been reported (*1–3*).

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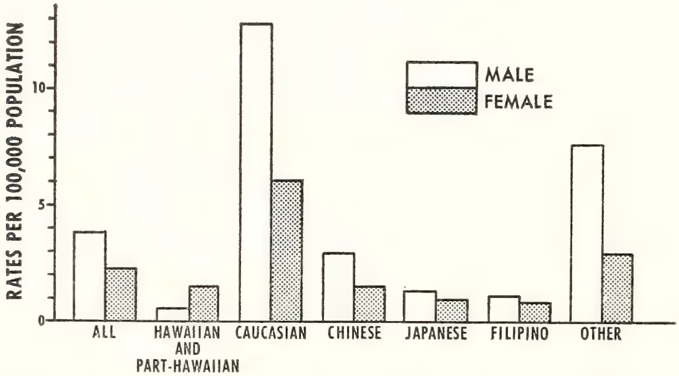
[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

MORBIDITY STUDY

In a cancer morbidity study conducted in Hawaii during the 7-year period between 1947 and 1954, the hospital records of 1,991 male cases of cancer and 1,680 female cancer cases were reviewed. A summary by sex and race of the cases of skin cancer included in this study will be found in table 1. There was microscopic confirmation of the diagnosis in 98 percent. Of the 79 males, 47 were Caucasians and 16 were Japanese. There were only 2 Hawaiian and part-Hawaiian men. Among men, the over-all rate per 100,000 population was 3.9, whereas the Caucasian rate was 12.7, the Japanese rate 2.2, and the Hawaiian and part-Hawaiian rate 0.6. There were only 40 women who had skin cancer. Twenty-four of these were Caucasians, 6 were Japanese, and 5 were Hawaiians and part-Hawaiians. This made a rate per 100,000 among women of 2.2 for all races, 6.1 for Caucasians, 0.8 for Japanese, and 1.4 for Hawaiians and part-Hawaiians. These data are shown graphically in text-figure 1. The Caucasian rate was more than 3 times that for all other ethnic groups combined, almost 6 times the Japanese rate, and more than 12 times the Hawaiian and part-Hawaiian rate in men. In women the ethnic differences were about the same as in men except that Caucasian women had a rate that was only a little more than 4 times that of Hawaiian and part-Hawaiian women. Even though the number of cases was relatively small, the rates for men in most ethnic groups except Hawaiian and part-Hawaiian were about double the rates for women. In the latter group the rate for men was less than half the rate for women.



TEXT-FIGURE 1.—Hospitalized skin cancer patients and rates per 100,000 population in Hawaii by sex and race, 1947–54.

TABLE 1.—Hospitalized skin cancer patients and rates per 100,000 population in Hawaii by sex and race, 1947–54

Ethnic groups	Males		Females	
	Cases	Rates	Cases	Rates
All	79	3. 9	40	2. 2
Hawaiian and part-Hawaiian	2	0. 6	5	1. 4
Caucasian	47	12. 7	24	6. 1
Chinese	4	3. 0	2	1. 6
Japanese	16	2. 2	6	0. 8
Filipino	4	1. 2	1	0. 7
Other	6	7. 4	2	2. 8

In 61 percent of the patients in this study, the lesions were on the face and head (excluding the lips). In 18 percent the cancers were on the extremities. Even though the number of cases was small, it might be of interest for completeness for one to compare the frequencies of cancer of the lip in the different ethnic groups by sex and race. There were only 9 men in this study who had cancer of the lip; 8 were Caucasians and 1 was Japanese. There were no cases among any of the other ethnic groups. Among women there were only 2 who had cancer of the lip; these were Caucasian women. Microscopic confirmation of the diagnosis was obtained in 92 percent of the cancer of the lip cases. The lesions were about equally divided between basal-cell and squamous-cell carcinomas. Melanomas were not included in this study.

SPECIAL COOPERATIVE PROJECT

In 1955 and 1956 a special study of skin cancer was conducted as a cooperative effort of private physicians and the Hawaii Territorial (now State) Health Department. Allison and Wong reported on this previously (*4*). The study was limited to the County of Honolulu where the major medical facilities of the State are centered. During this study, 293 cases of skin cancer were reported by dermatologists to a central register in the Department of Health. Table 2 is a summary of the cases and rates by ethnic groups in this project. No breakdown was made by sex in this study. Of the 293 cases, 276 were Caucasians, while 17 were

TABLE 2.—Skin cancer cases and rates per 100,000 population by race, Honolulu County, 1955 and 1956*

Ethnic groups	Cases	Rates
Caucasian	276	138. 0
Non-Caucasian	17	3. 1
Korean	2	17. 8
Chinese	3	4. 8
Japanese	9	3. 5
Hawaiian and part-Hawaiian	2	1. 6
Filipino	1	1. 4

*For source of data *see* (*4*).

non-Caucasians. Only 9 were Japanese and 2 were Hawaiians and part-Hawaiians. The rate for Caucasians was 138 per 100,000 population, while the rate for non-Caucasians was 3.1. The Japanese rate was 3.5, while the Hawaiian and part-Hawaiian rate was 1.6. These rates are shown graphically in figure 1.

There was microscopic confirmation in 93 percent of the 293 cases included in the study. Thirty-seven persons had more than one lesion of the same histologic type present at the time of reporting. Forty-four patients had 2 or more types of lesions at one time or another. All were Caucasians except 1 who was Caucasian-Hawaiian.

It was found in this study also that the body area most frequently involved was the head and face. The types most often found were squamous-cell and basal-cell carcinomas. There were 4 malignant melanoma cases. The rates by type in the different ethnic groups will be found in figure 2. It is especially interesting for one to compare the basal-cell and squamous-cell carcinoma rates in each of the 3 main ethnic groups, *i.e.*, Caucasians, Japanese, and Hawaiians and part-Hawaiians.

CAUCASIAN RATE

When Caucasian rates in Hawaii are compared with those of selected areas in the mainland United States, it becomes apparent that the rates in Hawaii are considerably higher. Allison and Wong (*4*) noted this in their report referred to previously. It was shown in the 1955–56 study that Honolulu had a skin cancer rate of 138 per 100,000 population as already stated, while Dallas, Texas, had a rate of 109 in 1948 as reported by Marcus (*5*); San Francisco had a rate of 90 in 1947 as stated by Gradowitz (*6*); and Philadelphia had a rate of only 39 in 1948 as reported by Cutler and Marcus (*7*). These rates were all among Caucasians. A graphic presentation of these comparisons will be found in figure 3. The morbidity study conducted between 1947 and 1954 was not used for comparison because it included only hospitalized patients. The other studies referred to included all cases reported in the areas concerned.

DISCUSSION

Although no data above reproach are available in regard to sampling, there seems no doubt that skin cancer is considerably more frequent among Caucasians living in Hawaii than among Orientals, the comparative rates being as high as 138 per 100,000 for Caucasians and 3.1 for non-Caucasians, and that this is due principally to the difference in pigmentation of the skin.

In 1946 Arnold (*8*) made some interesting observations concerning ethnic differences in skin cancer in Hawaii. In reporting on dermatoses in

Hawaii, he commented on the apparent low incidence of epithelioma of the skin in Japanese. He surveyed other physicians practicing in Hawaii at that time for their opinions regarding the incidence of skin cancer in Japanese. They all agreed that the condition must be quite unusual because they had seen very few cases. Arnold felt that the reason for this low incidence was not clear and was not merely a matter of pigmentation of the skin because Japanese are no more pigmented than Chinese and less so than Hawaiians and part-Hawaiians, yet he felt these latter ethnic groups were not immune to this type of cancer. He also stated that he did not think it was a matter of exposure to sunlight since the Japanese have fished and farmed in Hawaii for a long time.

In a morbidity study conducted by the State Health Department in cooperation with the Hawaii Medical Association during the 7-year period from 1947 to 1954, ethnic differences in skin cancer were shown. Even though the number of cases in the study was small and no scientific sampling procedure was followed, the ethnic differences appear to be quite definite. A study made by the dermatologists and the Hawaii Health Department during the years 1955 and 1956 also showed even greater ethnic differences.

Mortality studies have been made of skin cancer in Hawaii, but the number of deaths is so small that no significant statements can be made. A special effort was made to study Kaposi's sarcoma since the incidence of this type of tumor has been shown to be relatively high in some pigmented ethnic groups in other parts of the world. It has not been possible to show any ethnic differences in the incidence of this cancer in Hawaii. No cases could be found in the data collected during these studies. Mortality data were reviewed and no cases found.

Studies in Hawaii seem to confirm the work that Blum (*9*) has reported showing evidence that the high incidence of cancer in some parts of the world is not due to total sunlight but to carcinogenic radiation, and it was shown that this carcinogenic type of radiation is relatively more abundant in the lower latitude areas. (Hawaii is about 21 degrees north latitude. Sunshine is abundant throughout the year and the temperature ranges between 65 and 85 degrees Fahrenheit.)

Blum (*9*) has stated further that there is another factor that may help to explain the variation in skin cancer incidence with latitude. In the laboratory, rest periods from exposure to ultraviolet radiation lengthen the time to tumor appearance in mice. This suggests that the winters of higher northern latitudes constitute rest periods from carcinogenic radiation. In lower latitude areas such as Hawaii, there would be no real rest periods. It is possible also, Blum stated, that heating of the skin by the sun enhances the effect of the ultraviolet radiation.

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PLATE 19

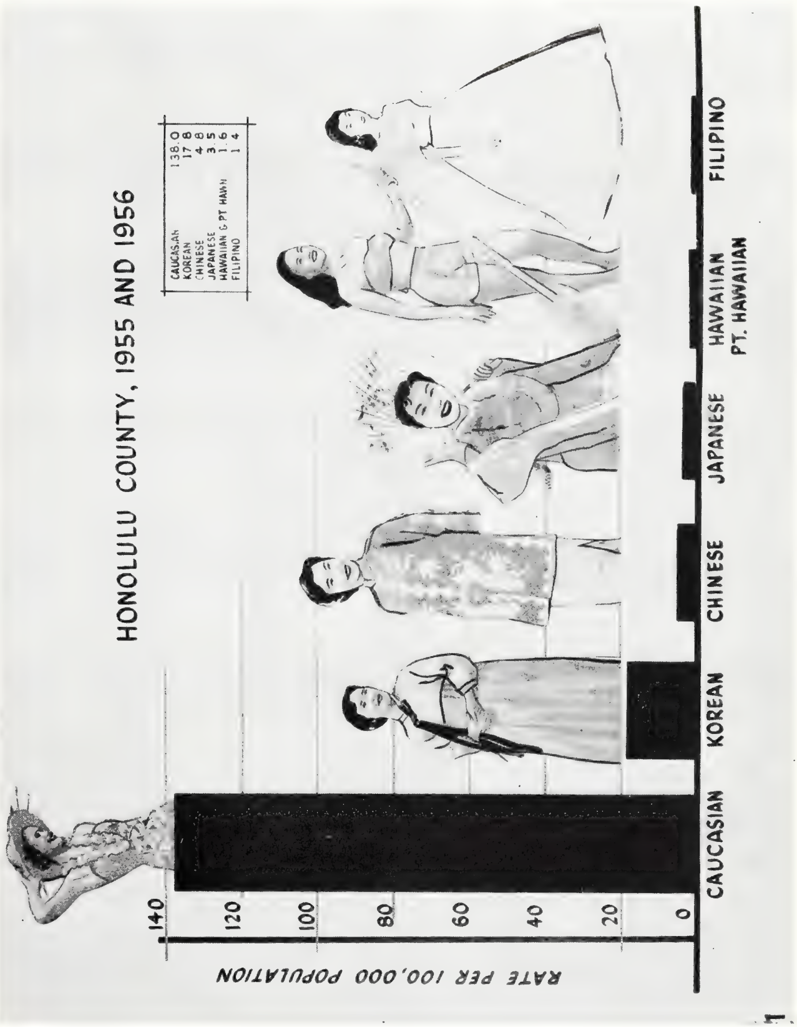
FIGURE 1.—Skin cancer annual incidence rates per 100,000 population by race.

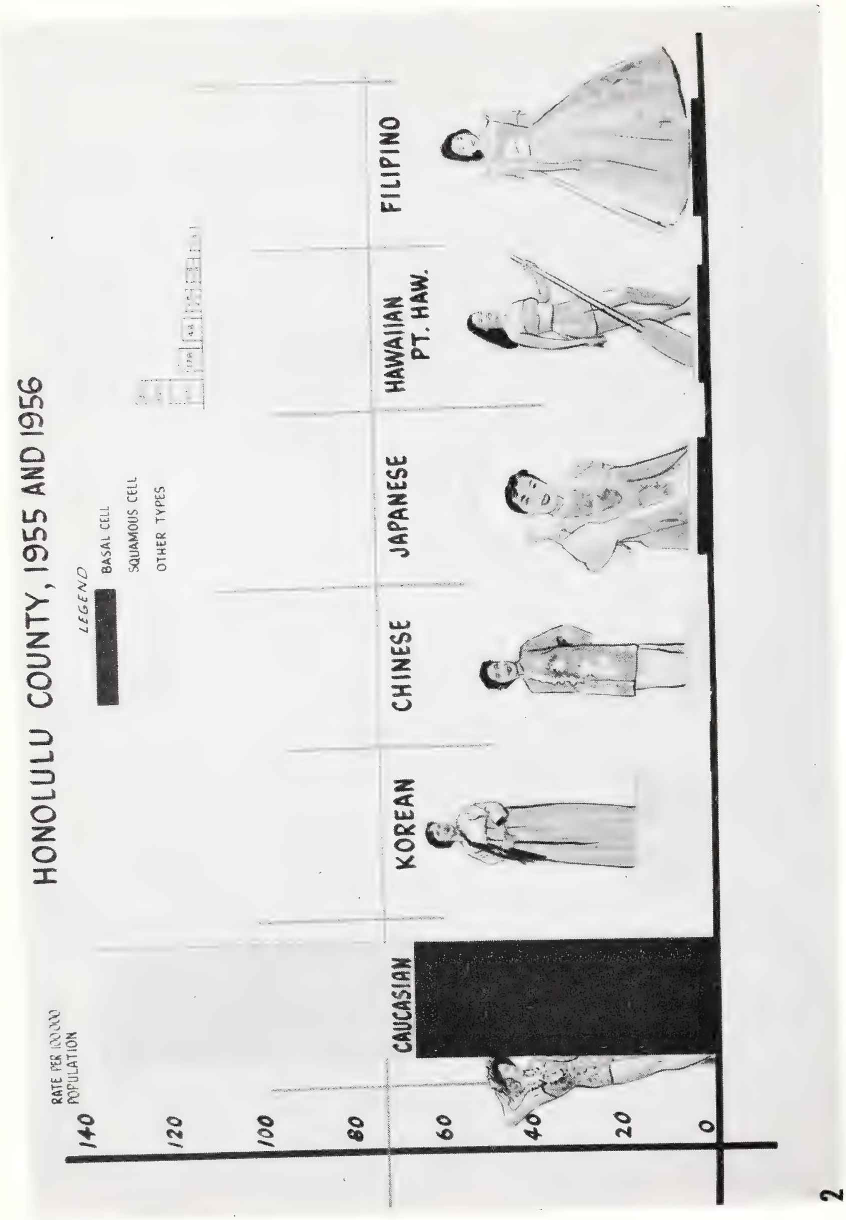
PLATE 20

FIGURE 2.—Skin cancer annual incidence rates per 100,000 population by race and type of cancer.

PLATE 21

FIGURE 3.—Skin cancer annual incidence rates per 100,000 white population.


HONOLULU COUNTY, 1955 AND 1956
CAUCASIAN 138.0
KOREAN 17.8
CHINESE 4.8
JAPANESE 3.5
HAWAIIAN & PT HAWN 1.6
FILIPINO 1.4
RATE PER 100,000 POPULATION
140
120
100
80
60
40
20
0
CAUCASIAN
KOREAN
CHINESE
JAPANESE
HAWAIIAN
PT. HAWAIIAN
FILIPINO



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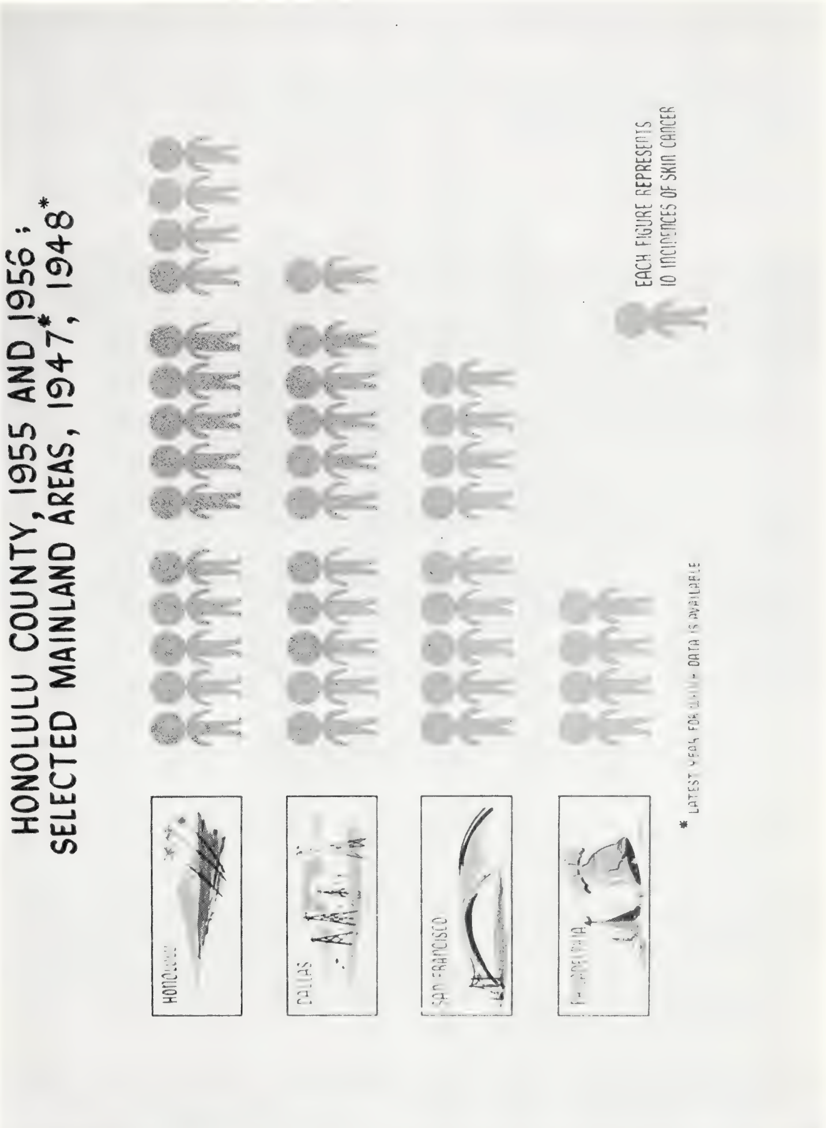
QUISENBERRY


HONOLULU COUNTY, 1955 AND 1956;
SELECTED MAINLAND AREAS, 1947*, 1948*
HONOLULU
DALLAS
SAN FRANCISCO
PHILADELPHIA
EACH FIGURE REPRESENTS
10 INCIDENCES OF SKIN CANCER
* LATEST YEAR FOR WHICH DATA IS AVAILABLE

3

SKIN CANCER IN INDONESIA[1]

SUDARTO PRINGGOUTOMO, M.D., and SUPRAPTI PRINGGOUTOMO, M.D., Institute of Pathology, School of Medicine, University of Indonesia, Djakarta, Indonesia

SUMMARY

Five hundred and twenty-six patients with skin cancer were examined at the Institute of Pathology in Djakarta during the last 6 years (1955–60). There were 225 squamous-cell carcinomas, 170 basal-cell carcinomas, 103 malignant melanomas, 20 penile squamous-cell carcinomas, 3 dermatofibrosarcomas, and 5 senile keratoses. Our findings regarding skin carcinoma occurring on the face in Indonesians were similar to earlier figures. Earlier observations on skin carcinoma in Indonesians showed that this cancer was more frequent in the lower limbs, especially the lower legs and feet, as compared with this site distribution in white people. Although still consistent with these earlier observations, data obtained in the present study showed a marked decrease of the site distribution of skin carcinoma in the lower legs and feet. Malignant melanoma, although still more prevalent on the feet than on other parts of the body, was also found to be less frequent than seen by earlier observers. The site distribution of malignant melanomas was also found to be decreasing from the foot as compared with data obtained 20 years ago. This tendency of skin carcinoma and malignant melanoma to recede from the lower limbs might be due to the fact that less people were going barefooted and to improved social and hygienic conditions in general. Our present survey showed also that there was almost no difference in incidence regarding the site distribution of skin carcinoma and malignant melanoma on the head (including face and ear) and neck among Indonesian males and females. However, many more cases were found occurring in the lower limb in males than females. The present survey also suggested that carcinoma of the penis occurred primarily in the noncircumcised group of the population.—NCI Monogr. No. 10: 191–195, 1963.

VOS, IN 1932, pointed out the strikingly high frequency of carcinoma of the skin, compared with other malignancies, in native Indonesians (*1*). He also found that in those natives skin cancer was less frequent on the face than on the other parts of the body. At the Netherlands-Indian Cancer Institute in Bandung, Vos found only 46.8 percent of all skin cancers localized on the head (*2*). Later observations by ten Seldam from this same Institute gave a similar figure (*3, 4*). Of 893 cases, 35.5 percent were localized on the face and 45.1 percent on the head. Ten Seldam also found that carcinomas of the leg were far more frequent in the lower age group.

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

The data compiled for this presentation and based on the examination of surgical specimens received by the Institute of Pathology in Djakarta during the last 6 years (1955–60) showed the same basic pattern as seen by former observers, but differed in some respects. These surgical specimens were received from almost all Indonesia. Lack of follow-up and clinical data made conclusive observations sometimes impossible.

Rukmono and Loa Wan Koen, in their survey of malignant tumors in Indonesians during 1952–59, found squamous-cell carcinoma of the skin comprising 8.7 percent of all malignancies and basal-cell carcinoma 2.7 percent (*5*). Squamous-cell carcinoma had been found slightly more in males than in females (10.5 and 7.2%, respectively), while there was about the same percentage for both sexes for basal-cell carcinoma.

The present study comprised 225 squamous-cell carcinomas of the skin (penile cancer not included), 170 basal-cell carcinomas, 103 malignant melanomas, 20 squamous-cell carcinomas of the penis, 3 dermatofibrosarcomas, and 5 senile keratoses.

Squamous-cell carcinoma was found more frequently than basal-cell carcinoma, which agreed with the findings of Vos and ten Seldam. Of 225 cases, 143 were found in males, 81 in females, and in 1 case the sex was unknown. One hundred and eighty-eight were found in Indonesians, 29 in Chinese, 7 in Europeans/Americans, and 1 in an Arabian. In regard to the age incidence, the high frequency was found at the age group of 31 to 40 years and a relatively similar frequency was noted in the older age groups. Histologically, most of the carcinomas showed cornification, more in males than in females. Grade I carcinoma was observed in 10 cases (8 Indonesians and 2 Chinese).

Carcinoma on basis of burn scar was found in 3 cases, one of which occurred 8 years after the burn. Multiple carcinomas were found in a Chinese male of 52 years. He was known to have hyperkeratotic plaques on both palms for 10 years, before which he presumably used arsenical drops. There was 1 squamous-cell carcinoma developing on a basis of lupus vulgaris, a rare lesion in Indonesia, with an eroding ulcer on the face, palate, maxilla, and teeth of 1-year duration. We found only 1 skin carcinoma that developed on a framboesia scar. Although interesting, the number of carcinomas arising in pre-existing lesions was too small to make a discussion possible.

Basal-cell carcinoma was found in 170 cases. Ninety-one were in males, 79 in females. There were 115 Indonesians (54 males), 23 Chinese (10 males), 26 Europeans (23 males), and 5 Americans (3 males); there was one whose race was unknown. The incidence was slightly higher in females than males among Indonesians. The age distribution showed that the highest incidence was in the 41- to 50-year age group and was relatively the same in the older age group. One hundred and forty-five cases, or 85.3 percent, were localized on the head and face. The inci-

TABLE 1.—Incidence of squamous-cell and basal-cell carcinoma on the face among Indonesians

	Face (including nose and eyelids)			
	Squamous-cell carcinoma		Basal-cell carcinoma	
	ten Seldam	S. Pringgoutomo	ten Seldam	S. Pringgoutomo
Men	64 (37.9%)	28 (38.4%)	105 (62.1%)	45 (61.6%)
Women	44 (33.3%)	25 (32%)	88 (66.7%)	53 (68%)
Total	108 (35.9%)	53 (35.1%)	193 (64.1%)	98 (64.9%)

TABLE 2.—Localization of squamous-cell and basal-cell carcinoma among Indonesians

Localization	Bandung, ten Seldam 14½ years		Djakarta, S. Pringgoutomo 6 years	
Face (around nose and eyelids)	301	35.5%	151	51.2%
Ears	16		15	
Scalp	86		39	
Neck	45		11	
Axillary and inguinal areas	13		2	
Trunk	76		32	
Arms	21		5	
Hands	12		3	
Upper legs	19		9	
Lower legs	220	34%	36	17.6%
Feet	84		21	
Total	893		324	

TABLE 3.—Distribution of skin malignancies of known localization and sex among Indonesians

	Male	Female
Squamous-cell carcinoma		
Head and neck	34	42
Lower limb	34	14
Basal-cell carcinoma		
Head and neck	48	55
Lower limb	2	—
Malignant melanoma		
Head and neck	10	11
Lower limb	42	21

dence on the cheek and eye region was 28 percent, on the nose 20 percent, and the forehead 9 percent. Basal-cell carcinomas arising in the cheek and eye region showed an equal sex distribution among Indonesians. Those on the forehead, however, were approximately twice as frequent in females as in males, and women also had more carcinomas on the nose. Histologically, approximately one third of the cases were of the solid type, while the pigmented and the keratotic types were each about one sixth of all the cases.

Malignant melanoma, as observed before by Vos, S. Hardjosoekatmo, ten Seldam, and others, was far from rare. In our series there were 103 cases, 83 occurring on the skin other than the eye region and 20 in the region of the eye.

The peak incidence lay in the 41- to 50-year age group, as found also by S. Hardjosoekatmo (*6*). However, there was an increased incidence of the 51- to 60-year age group.

It was shown again that the number of malignant melanomas of the foot was still slightly higher than in other body regions combined, though not as high as observed by earlier investigators. Of the 103 cases, 63 were found in males, 39 in females, and 1 in a person of unknown sex. There was an equal sex distribution of the melanomas in the eye region (male 10, female 10). Most of the melanomas were found in Indonesians (97), while only 4 were in Chinese and 2 in Europeans.

As mentioned before, the peak incidence occurred in the age group 41 to 50 years, which comprised 34 cases, or 33 percent. The oldest was 80, the youngest 9, and the average age was 47.4 years, the same as was observed by Hardjosoekatmo.

Histologically, most of the melanomas were carcinomatous. Pseudoepitheliomatous hyperplasia was seen in 2, while there was a definite malignant change from a nevus in 3. Thirty-one showed ulceration. Multiple malignant melanomas were observed in 3 instances, 1 occurring on the sole of the left foot and the left upper arm, another on the right foot, the inferior right eyelid, the left upper arm, and thorax, and another affecting the entire body, especially the left axilla. One of the malignant melanomas found in the eye region occurred in a pterygium of 3 years' duration, and histologically it showed a pigmented nevus that had become a malignant melanoma. Without other clinical data, 6 malignant melanomas were observed on the eyelid, 4 on the conjunctiva bulbi, 4 on the conjunctiva tarsalis, 1 in the caruncula, 2 in the bulbus oculi, and 2 in the eye. Ten metastases were found in the submitted lymph-node specimens. Although trauma was always difficult to evaluate as a possible etiologic factor, it is worth while to mention that 5 cases in

TABLE 4.—Malignant melanoma among Indonesians

	Netherlands East-Indian Cancer Institute, Bandung				Institute of Pathology, University of Indonesia, Djakarta	
	S. Hardjosoekatmo		ten Seldam		S. Pringgoutomo	
Eye	—		—		13	
Head and neck	10		22		9	
Body	4		5		8	
Arms	5		5		—	
Thighs	6		6		9	
Lower legs	6		10		1	
Sole of feet	73	72%	85	68.3%	38	55.7%
Toes	12		14		10	
Rest of feet	33		41		6	
Rest of body	15		17		3	
Total	164		205		97	

our material had a definite clinical history of trauma, 4 of which occurred on the sole of the foot due to injury by iron nails and wooden splinters, the other in the eye due to injury by a rice leaf.

Penile squamous-cell carcinoma was found only in 10 Indonesians, 8 Chinese, and 2 Europeans. The available clinical data showed that the majority of cases occurred in the foreskin, then in the glans penis, and the collum glandis. Unfortunately no reference to circumcision was made in the clinical history of these patients, but from their residences and names, it may be assumed that they were non-Islamic and therefore not circumcised.

Kouwenaar (*7*) found 35 cancers of the penis among 231 tumors in Chinese patients, or 15.1 percent, and only 3.7 percent in the circumcised Indonesians (Javanese). In our material we found penile carcinoma comprising 12.5 percent in Chinese and 2.4 percent in Indonesians.

Dermatofibrosarcoma was observed in 1 Indonesian male of 40 and 2 Indonesian females 30 and 23 years of age, occurring on the back, the lower abdominal wall, and the right thigh. The latter showed a recurrence 2 years after removal.

Senile keratosis was encountered in 5 males, 2 in Indonesians 101 and 55 years of age, and 3 in Chinese 43, 55, and 65 years old. The keratoses among Indonesians were localized on the left cheek and the chest above the sternum, while those of the Chinese were localized on the eyelid, the right temporal region, the face, chest, and back. Two of the 5 showed signs of malignancy.

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SKIN CANCER IN AFRICA[1, 2]

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SUMMARY

Information relating to skin cancer in Africa, gathered from various sources, indicates that whites have higher rates of skin cancers than the pigmented races, particularly squamous-cell and basal-cell carcinoma and melanoma. Adnexal tumors show no obvious racial differences. The high rates observed in exposed areas of whites and albino Bantu are mainly attributable to sunlight, as is indicated by the degree of actinic degeneration of the dermis. In the pigmented races, however, sunlight is a minor factor, and additional etiologic factors, such as trauma and chronic ulceration, are involved. This is seen in the frequency of these tumors on the lower limb. Keratoacanthoma and basal-cell carcinoma are exceedingly rare in deeply pigmented races. Estimates are provided of the frequency of albinism in South African Bantu and the incidence of skin cancer in these albinos, as well as the frequency of nevi in trunk skin of the pigmented Bantu.—NCI Monogr. No. 10: 197–214, 1963.

BY ITS sheer abundance, African experience adds weighty confirmation of current views on the etiology of skin cancer and provides certain quantitative measures of risk that may make possible the clearer definition of causal agents. The quality of the material varies with the state of development of cancer registration. For most areas the only statistics are ratios in clinical or histological series, but elsewhere we have death certificates or careful studies of morbidity. Striking differences are apparent between races as well as within races on this Continent.

In the following discussion, Kaposi's sarcoma has been omitted, since it is dealt with elsewhere (*1, 2*), but figures are included for cancer of the lip mucosa, penis, vulva, and anus, though these are treated separately from the remaining cancers of the skin.

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MORBIDITY RATES

The only published skin cancer morbidity figures available for Africa apply to the Johannesburg Bantu (*3*), where the observed cases were compared with the number expected by indirect standardization to the Johannesburg Bantu population. These figures have been recalculated (table 1) to fit the standard African population, 100,000 (*4*) (table 2).

TABLE 1.—Incidence of skin cancer standardized to the African standard population, 100,000, in Johannesburg, 1953–1955

International List Nos., 6th revision	Site	Males			Females		
		African	U.S. white	U.S. Negro	African	U.S. white	U.S. Negro
140	Lip	0. 2	3. 1	0. 3	—	0. 5	0. 1
190, 191	Total skin	1. 7	30. 0	2. 6	3. 0	22. 1	3. 4
190	Melanoma	0. 6	—	—	1. 3	—	—
191	Other skin	1. 0	—	—	1. 7	—	—

Incidence of skin cancer in Lourenço Marques standardized with indirect standardization to the Lourenço Marques population (approximately one half the size of the African standard population of 100,000)

International List Nos., 6th revision	Site	Males African	Males U.S. white	Males U.S. Negro	Females African	Females U.S. white	Females U.S. Negro
140	Lip	—	1. 29	0. 13	—	0. 18	0. 05
190, 191	Total skin	2. 6	12. 52	1. 20	1. 8	8. 48	1. 40

TABLE 2.—Recommended African standard population (*4*)

Age in years at last birthday	Number of persons
0	10, 000
5	10, 000
10	10, 000
15	10, 000
20	10, 000
25	10, 000
30	10, 000
35	10, 000
40	5, 000
45	5, 000
50	3, 000
55	2, 000
60	2, 000
65	1, 000
70	1, 000
75+	1, 000

In the Johannesburg Bantu residents, skin cancer rates are close to those of the U.S. Negro, but somewhat lower—which reflects possibly the benefits of deeper pigmentation in the Johannesburg Negroes (all of whom were pigmented, with the exception of 1 albino female with squamous-cell carcinoma).

In unpublished figures from Lourenço Marques the incidence of skin cancer is higher (*5*), as measured by indirect standardization to the Lourenço Marques population of 53,775 males and 45,260 females. The cases observed per annum are still much fewer than expected from U.S.

whites, but are nevertheless higher than those expected from an identical population of U.S. Negroes. The Moçambique rates probably correspond more closely to the incidence of skin cancer in tropical areas than do the Johannesburg figures.

MORTALITY RATES

Skin Cancer

Mortality statistics are available for the white, colored (mixed), and Asian (predominantly Indian) populations of South Africa (table 3). These figures have been analyzed for 1949–58 inclusive (for Asians the figures for 1949 are unavailable). The mean populations during these periods were 1,389,374 male and 1,389,479 female whites, 595,228 male and 599,035 female coloreds (mixed non-Negro), and 214,111 male and 193,121 female Asians. In 1958 medical certification of the cause of death was obtained in 99 percent of all white deaths, 88 percent of colored, and 98 percent of Asian deaths. The certification was slightly poorer in earlier years. The results are subject to the usual defects of death certificates, but for superficial cancers they should be reasonably accurate.

TABLE 3.—Skin and lip cancer mortality per 100,000 per annum standardized to the U.S. population, 1950: South African whites (1949–58), coloreds (1949–58), and Asians (1950–58) compared with the U.S. whites and Negroes (1955)

	Whites	Coloreds	Asians	U.S. whites	U.S. Negroes
		Skin cancer (190, 191)*			
MALE					
Deaths	524	70	5		
Rate	4. 71	2. 35	0. 80	2. 7	1. 3
FEMALE					
Deaths	344	55	8		
Rate	2. 74	1. 82	1. 56	1. 8	1. 0
		Malignant melanoma of skin (190)*			
MALE					
Deaths	187	21	—		
Rate	1. 59	0. 72	—		
FEMALE					
Deaths	165	14	2		
Rate	1. 33	0. 51	0. 25		
		Skin—other malignant neoplasms (191)*			
MALE					
Deaths	337	49	5		
Rate	3. 15	1. 63	0. 80		
FEMALE					
Deaths	179	41	6		
Rate	1. 45	1. 33	1. 31		
		Lip (140)*			
MALE					
Deaths	86	14	—		
Rate	0. 80	0. 54	—	0. 4	0. 1
FEMALE					
Deaths	8	4	2		
Rate	0. 06	0. 17	0. 35	0. 1	0. 0

*International List Nos., 6th revision.

In whites the mortality rate from skin cancer is very high and approaches that of Australia, where in 1941–45 the mortality rate in males was 5.2 and in females 3.0 per 100,000 (*6*). In the more pigmented mixed group the mortality is much lower, and in the Asians, most of whom are deeply pigmented Indians, it is as low as in the U.S. Negro (*7*). This high mortality in whites is attributable to sunlight; furthermore many South African whites consult quacks. It is unlikely that these differences can be explained by exposure to industrial carcinogens, since the pigmented races are more likely to be employed in the dirtier occupations. Furthermore, whites are more likely to seek treatment earlier than the other races, and a higher cure rate in whites is to be expected.

The secular changes in skin cancer mortality in whites (International List Nos. 190, 191) are noteworthy. In 1949 there were 58 male and 26 female deaths—the standardized rates per 100,000 being 5.7 and 2.4, respectively. In 1958 there were only 33 male and 29 female deaths, with standardized rates of 2.8 and 2.0, respectively—a fall in the standardized rates of 2.9 in males and 0.4 in females. This probably reflects improvement in treatment, as most people are still ignorant of the need for protection from sunburn.

In regard to mortality from "other malignant neoplasms of the skin," (International List No. 191), the racial differences in females are small, whereas in males there are marked differences. This probably reflects sex difference in exposure to sunlight, which makes the protective influence of pigmentation much more obvious in males.

For the mortality from melanomas (International List No. 190), on the other hand, the pigmented races show lower rates in both sexes. This suggests that, in addition to the factors of sunlight and exposure, a third factor may also be operating, *e.g.*, the frequency of junctional nevi.

Lip Cancer

Standardized mortality rates for lip cancer are presented for comparison. Marked variations are apparent in the male : female ratio in the different races: 13.3 in whites and 3.2 in coloreds exceeds that of skin cancers 2.2 in whites and 1.2 in coloreds. This suggests the operation of additional causal factors with a pronounced sex difference in the etiology of lip cancer, *e.g.*, smoking.

By comparison with mortality in U.S. whites, lip cancer rates in South African whites are elevated to the same degree as are rates for skin cancer. The South African preponderance of lip and skin cancers in whites by comparison with U.S. whites is easily explicable on differences in exposure to sunlight.

HISTOLOGICAL RATIOS

From most of Africa only ratios of cancers in histological series are available. The proportion of skin cancers is likely to be abnormally inflated by greater accessibility to biopsy, as well as the over-all reduction in cancer incidence in many sites in primitive races compared with westernized races.

The results of such studies (table 4) reveal a uniformly high incidence of squamous-cell carcinoma throughout Africa, a somewhat fluctuating but low proportion of basal-cell growths (probably related to variations in diagnostic criteria for distinguishing basal-cell carcinomas from adnexal growths), and remarkable fluctuations in the proportions of malignant melanomas.

Previously unpublished histological ratios from the Johannesburg survey of 1953–55 (*3*) enable comparison of Johannesburg residents with Johannesburg patients who were nonresidents (and hence somewhat less urbanized) and a group of rural cases from the Northeastern Transvaal (table 4). The rural cases resemble the rest of Africa, the nonresidents occupy an intermediate position, and the urbanized Johannesburg group shows a marked reduction in all 3 types of skin cancer.

TABLE 4.—Ratios of skin cancers in histological series in Negroids in Africa (both sexes combined)

Region	Reference	All cancers	Squamous-cell carcinoma		Rodent		Melanoma	
Tunisia	(*29*)	2, 818	256	9. 1	74	2. 6	31	1. 1
French West	(*30*)	1, 884	235	12. 5	24	1. 3	49	2. 6
Gold Coast	(*31*)	1, 193	166	13. 9	12	1. 0	63	5. 3
Nigeria	(*32*)	500						8. 0
Nigeria	(*33*)	1, 000	122	12. 2			62	6. 2
Uganda	(*34*)	734	100	13. 6	1	0. 1	13	1. 8
Kenya	(*26*)	546	64	11. 7			59	10. 8
Dar-es-Salaam	(*35*)	662	85	13. 0	10	1. 5	25	3. 8
Belgian Congo	(*36*)	2, 529	233	9. 2	16	0. 6	135	5. 3
Belgian Congo	(*37*)	94	15	16. 0			3	3. 2
Belgian Congo	(*38*)	88	12	13. 6	7	8. 0	5	5. 7
Transvaal	(*39*)	497	20	4. 0	6	1. 2	17	3. 4
Johannesburg								
Residents		693	9	1. 3	1	0. 1	12	1. 7
Nonresidents		564	27	4. 8	2	0. 4	19	3. 4
Rural		212	29	13. 7	3	1. 4	20	9. 4
Natal	(*40*)	1, 921	174	8. 9	7	0. 3	90	4. 6
Moçambique	(*41*)	1, 273	99	7. 8	29	2. 3	26	2. 0

TRANSVAAL HISTOLOGICAL SURVEY, 1949–53

The results of a more extensive study of histological ratios will be given. All Transvaal material received at the South African Institute for Medical Research between 1949 and 1953, inclusive, has been reviewed.

Skin Cancer in Whites

This material follows the usual pattern, the most common tumors being basal-cell carcinomas, followed by squamous-cell carcinomas, melanomas, and finally adnexal tumors. The importance of sunlight in the first 3 groups of tumors is suggested by their frequency on exposed areas and their association with severe grades of what has been regrettably misnamed "senile degeneration" of the dermis. African experience confirms fully that this is a reaction to prolonged exposure to sunlight and, apart from such exposure, is unrelated to senility. This change in exposed areas, its relatively greater development in blondes and, in particular, albinos compared with pigmented skins, and its absence from nonexposed areas in the same persons support the current view that this is not a senile change but a direct effect of actinic irradiation on the dermis. In this series of skin cancers it has been observed in a 10-year-old albino Bantu. By comparison, pigmented Bantu skin shows this change rarely, and then only to a mild degree.

To evade the question whether the material is actually elastic tissue or elastotic degeneration of collagen (although these opinions need not be conflicting), I shall use the term "actinic degeneration."

Grading of actinic degeneration ("senile elastosis").—For comparison of effective exposure of the dermis to sunlight, 4 grades of this condition have been distinguished: 1) basophilic staining of swollen fibers in the upper dermis; 2) more extensive basophilia, with droplet formation; 3) areas of confluent or amorphous colloid material between fibers, which represent less than 50 percent of the basophilic material; 4) areas of confluent amorphous material predominating in the regions of basophilia.

The confluent areas occasionally contain very refractile, highly eosinophilic granules. These are rare and do not warrant separate classification. The assessment of basophilic fibers should be carried out away from the cut edge of the tissue, since this may stain abnormally, the affinity for hematoxylin presumably being increased by a steel cutting edge.

Keratoacanthomas of lip mucosa.—In this series keratoacanthomas were found only in whites; 3 occurred in males on the lip mucosa. These were not followed in serial section, but were evidently sagittal sections through the center of the crater—an area of unaffected mucosa remained on the red margin and the tumor itself lay over the labial muscles. In addition to the characteristic histological appearance, the diagnosis was confirmed in all 3 patients by the history of rapid growth—6 weeks in one 40-year-old man and involved the upper lip, the other 2 on the lower lip, 1 of which grew in 4 weeks in a 41-year-old man and the other in 3 weeks in a man 69 years old.

A case of keratoacanthoma of the red margin of the lower lip has been reported by Whittle and Davis (*8*). These cases call in question the current opinion that the tumor arises only from existing hair follicles. Unfortunately the presence or absence of Fordyce's condition was not noted in the cases mentioned, so that the possibility has not been excluded that these mucosal sebaceous glands may have been involved.

The absence of keratoacanthomas in the pigmented Bantu in this series confirms the probable influence of sunlight in the development of these tumors. They do occur in Bantu, although rarely, *e.g.*, in 1959–61 at this Institute, 243 keratoacanthomas were diagnosed in whites and only 7 in Bantu, 2 being in females.

Skin Cancers in Pigmented Bantu

In any consideration of etiological influences in skin cancer, the high frequency of skin cancer in albinos and the relative frequency of albinism among the South African Bantu make it desirable for one to distinguish separately the groups with an inordinate susceptibility to skin cancer (table 5).

Among pigmented Bantu, squamous-cell carcinomas outnumber all others, mainly because of the large number on the lower limb (78/132 or 59 percent). These are associated for the most part with previous trauma, burn scars, and chronic ulcers.

Malignant melanoma follows, also with a high frequency (63/87 or 72 percent) on the lower limb, where the tumor develops predominantly on the soles of the feet and, presumably, is related to trauma from people walking barefooted. The proportion of melanomas of the foot in urban Bantu is much lower than that in rural Bantu (*3*), and the ratios in table 4 suggest that melanomas in consequence are much more common in rural Bantu.

Basal-cell carcinomas are extremely rare in the pigmented Bantu and are not as common as adnexal tumors. The few basal-cell carcinomas resemble those occurring in whites. In some instances, the relationship of basal-cell carcinomas to sunburn is probable as they also occur on exposed areas and often show a minor degree of actinic degeneration of the dermis.

Sweat-gland tumors, sebaceous-gland tumors, and trichoepitheliomas occur rarely, and it seems that the susceptibility of the Bantu to these growths is similar to that in whites.

Frequency of Nevi in Pigmented Bantu

The rarity of malignant melanomas in sites other than the feet in the pigmented races might be related to a general rarity of pigmented nevi. For an examination of the frequency of nevi, trunk skins were obtained from 11 Bantu cadavers, cleared in glycerin for 3 days at 37° C, and examined under bright transillumination. Every dark area greater than 1 mm was cut out and sectioned. The results are given in table 6.

Among the Bantu examined one quarter of all pigmented areas was keratotic follicles, and there were as many ephelides and lentigines. No junctional nevi were observed, and intradermal nevi formed only 3.4 percent. Mesodermal melanoblasts accounted for 12 percent, but were also commonly present in the lentigines. This high frequency of pigmented spots, consisting of mesodermal melanoblasts, and the low frequency of intradermal (4 cases) or junctional nevi are noteworthy, since the trunk skin accounts for 36 percent and if the rest of the body area were in proportion it would average 1 nevus per person.

The lighter skin pigmentation in South African Bantu than in the Central African group makes detection of nevi easier. Clinical impres-

TABLE 5.—Frequency of cases of skin cancer in the material from the Transvaal Bantu received at the South African Institute for Medical Research, 1949–1953*

Type	Sex	Unspecified	Head	Neck	Abdomen and pelvis	Thorax	Back	Fossae	Upper limb	Lower limb	Total
					Pigmented Bantu						
Squamous-cell carcinoma	Male	7	16	2	4	—	—	2	3	49	83
	Female	4	8	—	—	—	1	2	5	29	49
Basal-cell carcinoma	Male	2	5	—	—	1	—	—	—	—	8
	Female	3	2	—	—	—	—	—	—	—	5
Sweat-gland tumor	Male	4	9	1	1	—	1	—	3	1	20
	Female	1	5	1	—	—	—	—	4	2	13
Sebaceous-gland tumor	Male	—	2	1	—	—	—	—	—	—	3
	Female	—	1	—	—	—	—	—	—	—	1
Tricho-epithelioma	Male	—	2	—	—	—	1	—	—	—	3
	Female	—	1	1	—	—	—	—	—	—	2
Melanoma	Male	1	5	—	1	3	1	1	6	29	47
	Female	—	1	—	—	2	—	1	2	34	40
					Albinos						
Squamous-cell carcinoma	Male	—	5	—	—	—	1	1	2	—	9
	Female	—	4	1	—	—	—	—	—	—	5
Basal-cell carcinoma	Male	—	—	—	—	—	—	—	—	—	0
	Female	—	—	—	—	—	—	—	1	—	1
Sweat-gland carcinoma	Male	—	—	—	—	—	—	—	—	—	0
	Female	—	1	—	—	—	—	—	—	—	1
Melanoma	Male	—	1	—	—	—	—	—	—	—	1
	Female	—	—	—	—	—	—	—	—	—	0

*Penis: 31 squamous-cell carcinomas. Vulva: 28 squamous-cell carcinomas plus 2 malignant melanomas, 1 in a "partial albino." Lip mucosa: 5 males and 3 females pigmented with squamous-cell carcinomas; 2 albino males with squamous-cell carcinomas; and 1 albino male with melanoma. Anus: squamous-cell carcinomas, 3 males and 2 females. In addition to the melanomas reported here, there were inguinal-gland metastases of unstated origin in 2 males and 2 females (pigmented) and superficial mucosal melanomas in 2 males (lip and nose).

TABLE 6.—Histological analysis of pigmented areas on 11 trunk skins from Bantu cadavers

Type	Number	Percentage
Keratotic follicles	29	25. 0
Keratotic follicles with mesodermal melanoblasts	2	1. 7
Epidermal cysts (possibly keratotic follicles)	9	7. 8
Lentigines	28	24. 1
Ephelides	28	24. 1
Epidermal proliferation	2	1. 7
Mesodermal melanoblasts (blue nevi)	14	12. 1
Intradermal nevi	4	3. 4
Total	114	99. 9

sions confirm that, although nevi are familiar beauty spots, they are rare among the South African Bantu.

Skin Cancers in Albino Bantu

The high susceptibility of albino Bantu to skin cancer has been recognized in South Africa since 1911 (*9*) and is well substantiated in the Transvaal (*10*), where the altitude of the plateau rises to over 6,000 feet (2,000 m), as well as in Cape Town (*11*).

The albinos among South African Bantu resemble those in Nigerian Negroes (*12*). In the majority the iris is pigmented and the exposed skin, which becomes very wrinkled and hyperkeratotic, is often freckled. Following the terminology recommended by Fitzpatrick (*13*), they represent cases of generalized albinism in which some persistence of the ability to produce melanin is detectable in the iris and freckles, but the skin does not tan.

The skin cancers seen in albino Bantu in 1949–53 included, besides squamous-cell carcinoma, 1 basal-cell carcinoma, 1 melanoma, and 1 sweat-gland carcinoma. Other cases of the last 3 types of tumors in albinos are known, so that it seems that the incidence of all these has increased, though not to the same degree as squamous-cell carcinoma.

The relative frequency of these tumors in albino Bantu is indicated in table 5, where they represent 14 of 146 squamous-cell carcinomas (1 in 10.4 or 9.6 percent). The site distribution is naturally very different, all the squamous-cell carcinomas of albinos being on sites other than the lower limb. In these other sites the albino carcinomas represent 14 of 53 (1 in 3.8 or 26.4 percent).

One albino was encountered in 8 Bantu with basal-cell carcinoma (12.5 percent), and 1 albino with a melanoma of the skin of the lower lip in 87 Bantu with melanomas of the skin. (This figure does not include 2 male and 3 female Bantu with metastases in lymph nodes probably of skin origin.) This represents the fourth published case of an albino with a melanoma (*14–16*); there is a fifth one in the Transkei survey (not included here). It is probable that albinism is associated with an increased susceptibility to melanoma. The current percentage (1.2) of albinos with melanomas is altered markedly if one excludes melanomas of the lower limbs—the figure then becomes 1 in 24 (4.2 percent).

Ratio comparisons of this type are affected by the number of factors influencing the denominator, *i.e.*, the prevalence of conditions inducing skin cancers. Any change in the large proportion involving the lower limbs will alter the ratios.

Our material also included 1 "partial albino" with malignant melanoma of the vulva. This patient was reputed to have had areas of depigmented skin present from birth, but there was no history of albinism in his family. The patient was not seen personally, and if this were a case of localized albinism, it should have shown evidence of Mendelian dominant transmission. Sections from the depigmented areas showed pigmented active dendritic melanoblasts in the epidermis, which is unlike localized albinism and more suggestive of vitiligo, in which the melanoblasts are capable of recovery. The melanoma in this patient was heavily pigmented and arose from an area of pigmented skin, so that the localized albinism, if such, was not relevant.

Frequency of Albinism in South African Bantu

To the superficial observer it is obvious that albinism is much more common among the Bantu than among the other races in South Africa, and in 1960 Higginson and I (*3*) believed that its frequency was probably about the same as that reported by Barnicot for Nigeria; namely, 1 in 5,000 (*12*). Since albinos do not migrate in search of labor as often as normally pigmented Bantu, the measurement of the incidence of this condition in a native area is preferable to an urban region with its preponderance of migrant labor. I have attempted, therefore, to measure its frequency in the Ciskei and Transkei native reserves of the Cape Province, but as I did not achieve a complete survey the procedure followed is given in detail:

In 1958 I spoke about the need for such a survey to the Chief Magistrate of the Transkei, Mr. T. Ramsay, and the Bantu representatives of the Bunga, the Transkeian Territorial Authority. Subsequently the Chief Magistrate instructed all Native Commissioners to report albinos in their districts to the Cancer Research Unit in Johannesburg. A number failed to send in returns.

At the suggestion of Dr. R. J. W. Burrell, school principals were next enlisted and registered further cases. Social workers were also instructed to notify me as they met albinos in the course of their survey of esophageal cancer.

By September, 1959, 197 people had been registered, but many districts had reported no albinism, and it was evident that a full survey demanded a detailed study of each district, such as could be carried out by social workers during Dr. Burrell's survey of esophageal cancer. I have, therefore, sent Dr. Burrell my list in the hope that he may complete the survey. Meanwhile, the incomplete material permits estimates in certain districts

(Ciskei and Transkei), where registration seems to have been adequate. The combination of results for 2 adjoining Bantu areas, such as the Ciskei and the Transkei, is reasonable, since they are administrative divisions and both are occupied by Xhosa tribes. It seems unlikely that gross differences in gene frequencies will occur in this coastal strip, for studies of blood groups fail to reveal any tribal differences within South Africa (*17*) between tribes that have been separated by the barriers of language for centuries.

The results are given in tables 7 and 8. Table 7 shows the age distribution of all the albinos and the percentages, as well as the percentage age distribution in the Transkei for the total population at the 1951 census. (That for the 1960 census is still unobtainable.) The male : female ratio is 0.89, which is not significantly different from unity. The age distribution suggests that the group 0 to 9 years has been underregistered and that the actual figure should be about one and a half times as high as that recorded for this age group.

TABLE 7.—Age distribution of albinos registered from the Ciskei and Transkei compared with that for the Transkei in the 1951 census

Age group	Albino population		Both sexes (%)	Total population of both sexes (%)
	Male	Female		
0–9	20	20	20. 8	29. 7
10–20	21	26	23. 9	23. 4
20–30	10	19	14. 7	14. 3
30–40	12	15	13. 7	12. 1
40–50	11	14	12. 7	9. 1
50–60	7	3	5. 1	5. 9
60–70	4	6	5. 1	3. 4
70–80	3	1	2. 0	2. 2
80+	—	—	—	—
Unknown	4	—	2. 0	0. 1
Total	92	104*	100. 0	100. 0

*Plus one of unstated sex, aged 5 to 9.

The registrations in table 8 are shown by relative frequency rates for given magisterial districts, based on the population at the 1960 census. The population in 1960 will be somewhat larger than that of the surveys in 1958 and 1959, but since census estimates of Bantu populations are usually underestimates (*3*), this is no loss.

It will be seen that in the territories there are 4 groups: 1) Albinism exceeds 25 per 100,000 and rises as high as 64. These exceed Barnicot's estimate for Nigeria (*12*); 2) albinism exceeds 10 per 100,000 and, unless these are random low counts, one may suspect that registrations have been incomplete; 3) below 10 per 100,000 almost certainly indicates incomplete registration; 4) no albinos were registered. Some underregistration can be anticipated (unless infanticide of albino children is practiced). I have ignored these last 2 groups, therefore, and calculated the rates for albinism for group 1 (38.5/100,000 or 1 in 2,600) and for groups 1 and 2 combined (24.5/100,000). If one allows for underregistration in

Table 8.—Cases of albinism reported from the Ciskei and Transkei districts, 1958–1959, related to the 1960 census population

District	Male	Female	Total	Population	Rate
Ciskei					
Keiskammahoek	5	5	10	19,929	50.2
Glen Grey	5	5	10	77,846	12.8
Whittlesea	3	7	10	15,698	63.7
TOTAL	13	17	30	113,473	26.4
Transkei					
I. *Rates over 20 per 100,000*					
Mqanduli	11	6	17	53,547	31.7
St. Marks	13	16	29	62,982	46.0
Umtata	15	10	25	65,854	38.0
Kentani	7	10	17	52,572	32.3
Port St. John's	3	4	7	28,196	24.8
Total	49	46	95	263,151	36.1
II. *Rates over 10 per 100,000*					
Matatiele	5	5	10	68,281	14.6
Mount Ayliff	—	3	3	22,817	13.1
Tsolo	2	7	10*	47,497	21.1
Engcobo	8	4	12	80,067	15.0
Xalanga	1	3	4	25,699	15.6
Butterworth	3	1	4	31,036	12.9
Tsomo	2	3	5	37,042	13.5
Willowvale	3	9	12	66,005	18.2
Total	24	35	60*	378,444	15.9
III. *Rates below 10*					
Mount Currie	1	—	1	21,367	4.7
Umzimkulu	4	—	4	65,604	6.1
Idutywa	1	3	4	43,592	9.2
Lusikisiki	—	3	3	82,300	3.6
Total	6	6	12	212,863	5.6
TOTAL	79	87	167*		
IV. *No albinos or reports submitted* Mount Fletcher (52,817); Quembu (51,533); Mount Frere (56,962); Elliotdale (37,554); Nqamakwe (50,664); Bizana (66,173); Flagstaff (49,500); Libode (48,100); Ngqeleni (61,712); Tabankulu (55,200).					

*One sex not stated.

the age group 0 to 9, then the rate for the combined group becomes 26.6 per 100,000 (1 in 3,759). It is suggested that this last figure, although it probably underestimates the actual incidence of albinism, be regarded as the present estimate of its frequency.

Detailed genetic studies are lacking, but the condition behaves as a Mendelian recessive. In one family 2 albinos married (cases 77 and 78) and had 3 albino children, 1 female (case 79) and 2 males (cases 80 and 81). The genes for albinism in these parents were clearly allelic in contrast to the case reported by Knox (*18*).

For a homozygous recessive affecting 1 in 3,759 persons, and if one assumes random mating, the gene frequency is 1 in 30.7, *i.e.*, 0.033. The assumption that mating is random among the Bantu seems reasonable despite the lack of information on consanguinity, for tribal taboos on marriage with any member of the parent's or grandparent's clans are very strict. In rural areas marriage is not permitted if even the clan name is the same. These strictures are being relaxed with migration to the cities, and, as the habit of *ukumetsha* or *coitus interfemoris* is acceptable among the unmarried and nowadays permitted between members of the same clan, it is evident that opportunities for illegitimate consanguineous matings exist, though they would be very difficult to assess quantitatively.

The estimated gene frequency of 0.033 is thus likely to be fairly accurate. From consanguinity data, the gene frequency in Caucasians is estimated as 0.0021 to 0.0030 and in Japanese from 0.0029 to 0.0075 (*19*).

The incidence of albinism in the Ciskei and Transkei thus appears to be the highest recorded, apart from that noted in the San Blas Indians where the most recent figure is 33 in 2,780 or 1 in 187 (*20*). This is a small population, however, with considerable inbreeding (*21*), and infanticide of albinos was carried out in the past. A high rate of albinism does not necessarily occur throughout Africa, and in the Kivu area of the Congo Gigase (*22*) there were none in 50,000 people examined; on our rates 15 cases would have been expected. There are albinos, but they are evidently very rare.

The explanation for this high rate among the South African Bantu is still uncertain. It seems unlikely that this single characteristic would be subject to higher mutation rates in this group. Albinos have a higher death rate from skin cancer than do nonalbinos—at least in the Transvaal and in the Cape (*11*)—and it is difficult to imagine that among the Bantu the homozygotes have an increased advantage in survival over nonalbinos as compared with the Japanese or Europeans. Study of heterozygotes might indicate a selective advantage over the normal population. Certainly, if heterozygosity gives rise to a paler skin, this would have great selective value, as this is regarded as a mark of beauty.

Incidence of Skin Cancer in Transvaal Albino Bantu

Estimation of the incidence of cancer in albinos is possible from the number of cancers of distinct sites, observed in the Transvaal albinos for 5 years, to the frequency of albinos observed in the Cape Province Bantu.

These figures are subject to many assumptions:

1) That the proportion of cases examined histologically in the Transvaal was the same as in Dorn and Cutler's (*23*) survey (66.3%). The actual figure is perhaps much lower than this.
2) That in cases with multiple cancer, all sites were biopsied. This also is optimistic.
3) That albinism in the Transvaal is as frequent as in the Transkei. This seems a reasonable assumption.
4) That the expectation of life of the albino population is the same as that of the pigmented Bantu in the Transvaal. This is doubted by many clinicians.

The mean population of the Transvaal for 1949–53 is given by the 1951 census as 1,948,779 Bantu males and 1,534,991 females (total, 3,483,770). If one assumes the estimated rate of 1 albino per 3,759 persons, there were 518 male and 408 female albinos (total 926) at risk. The crude annual rates of squamous-cell carcinoma are thus 579 per 100,000 for males, 408 for females, and 504 for both sexes combined—figures which are approximately 10 times the incidence of skin cancer of all forms in U.S. whites, as reported by Dorn and Cutler. The rates for basal-cell carcinoma, melanoma, and sweat-gland carcinoma each are 36 per 100,000 for both sexes combined and, being based on single cases, must be regarded as approximate.

Skin Cancer in Bantu With Xeroderma Pigmentosum

In this series a 4-year-old girl, in whom a squamous-cell carcinoma had already developed, had xeroderma pigmentosum.

In contrast to albinism, xeroderma pigmentosum is a rare condition in the Bantu, and its gene frequency is probably not very different from that recorded in whites (0.0019–0.0026) or Japanese (0.0057–0.0075) (*19*). (If our case were the only one in the Transvaal during the 5 years, this would indicate a gene frequency of 0.0011.)

Although skin pigmentation in Bantu is deeper than normal, their susceptibility to skin cancers is apparently as great as in whites. Cancers develop in childhood much earlier than in albinos. Actinic degeneration of the corium is not present, which suggests that the ultraviolet radiation acts directly on susceptible cells and not via stromal changes, contrary to what Gillman *et al.* (*24*) have postulated.

The occurrence of malignant melanoma in association with xeroderma pigmentosum also supports the view that ultraviolet irradiation may induce malignant transformation in melanoblasts.

Absence of Skin Cancer With Vitiligo

In view of the increased susceptibility of albino skin to ultraviolet carcinogenesis, it is remarkable that we have no records of skin cancers associated with vitiligo.

In 30 years of practice as a dermatologist in the tropics and in South Africa, Loewenthal (*25*) has seen neither solar keratosis nor skin cancer in sites of vitiligo, whether in whites, Bantu, or Indians, though he has noted a skin cancer in a partial albino. This apparent immunity of vitiliginous skin deserves study. To some extent, of course, it may be explained by the shorter duration of vitiligo, but this seems inadequate for an entire explanation of the difference.

ETIOLOGIC FACTORS

Sunlight

African experience provides abundant evidence of the protection offered by skin pigmentation (in all but cases of xeroderma pigmentosum) and supports the hypothesis that assigns a major role to sunlight in the development of many squamous-cell carcinomas, keratoacanthomas, basal-cell carcinomas, and melanomas of exposed areas in whites and albino Bantu. Even in the pigmented Bantu, exposed areas bearing squamous-cell and basal-cell carcinomas often show actinic degeneration, which suggests that sunlight may be a factor here, though of lesser importance. The absence of skin cancer in sites of vitiligo remains puzzling.

Chronic Ulcers

Especially on the lower limb a high proportion of squamous-cell carcinomas occurs in association with chronic ulceration, which may be syphilitic, tropical, or one following burns or trauma. Tropical ulcers and veld sores are very rare in South Africa, but are exceedingly common in other regions.

At a time when excision was the routine therapy for tropical ulcers, Vint (*26*) discovered in 262 routinely excised ulcers 1 melanoma, 5 epitheliomas, and 3 which he regarded as definitely precancerous, *i.e.*, 2 percent were frankly malignant. Since in Kenya, in 1932, 18,000 patients with tropical ulcers were treated in the hospital, this rate would imply at least 360 cancers of the skin. For the Congo, Janssens *et al.* (*27*) reported that 9 percent of chronic tropical ulcers become malignant. Such figures are greatly influenced by the circumstances necessary to bring a patient to the hospital and the stage at which he is treated. Careful statistical studies are needed with special reference to the problems of prevalence, chronicity, and the incidence of malignant change. The relative immunity of varicose ulcers of the legs to malignant change also deserves quantitative study.

Trauma

That old healed scars tend to develop squamous-cell carcinoma is apparent from South African material. Many of these scars result from old burns, where combustion products and depigmentation, due to destruction of melanoblasts, may also be involved. Such burns are common in Bantu epileptics who fall into open fires on the ground or hut floors.

Trauma is also a factor in malignant melanomas of the soles of the feet. Junctional nevi are sometimes detectable at the edges of such growths, but generally the lesion is too advanced to discern if this change has occurred. Although a history of specific trauma is not obtained from these people and many lesions arise on the non-weight-bearing areas of the sole, a marked reduction in frequency of these tumors is noted in urban groups who wear shoes and walk on more even surfaces.

In regard to the rarity of melanomas at other sites, the infrequency of nevi among the Bantu may contribute to the lower rates observed.

Quack Plasters

Cancer plasters are still used by South African whites. These produce a very characteristic histological picture of necrosis with widespread infiltration with leukocytes. This factor contributes to mortality rather than to etiology.

Tobacco and Lip Cancer

The extraordinary differences in sex ratios of the mortality of white patients with cancers of the lip and skin suggest that other factors, notably tobacco smoking, are probably also responsible.

Viruses

No evidence for a viral origin of these skin cancers is available except for one woman in which typical condylomas of the vulva, presumably viral, progressed into squamous-cell carcinoma. This has also been reported from Johannesburg by Charlewood and Shippel (*28*).

Uncleanliness

African Negroid populations tend to wash themselves thoroughly, when possible, and do not display the high incidence of skin (dhoti and sari cancers), penile, and anal cancers seen in certain Asian groups. Nevertheless, the scarcity of hot water, soap, and toilet paper may be reflected in the incidence of certain of these cancers, *e.g.*, cancer of the anus.

The common practice of adult circumcision is probably responsible for some reduction in the incidence of penile cancer as compared with areas where circumcision is not practiced. Some of the improvements may be explicable simply on removal of susceptible epithelium, but some benefit probably results from adult circumcision, though it is evidently far less than that following circumcision in infancy.

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SKIN CANCER IN INDIA[1]

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SUMMARY

Cancer of the skin is relatively uncommon in Indians despite intense ultraviolet exposure. Special types of skin cancer, presumably due to extreme heat, combustion, and chronic friction are found as kangri, dhoti, and chutta cancer. The clinical features, course, and incidence of the various types of skin cancer in India are briefly discussed, and tentative suggestions as to their etiology are proposed.—NCI Monogr. No. 10: 215–223, 1963.

INDIA IS the seventh largest country in the world. It covers an area of 1,260,000 square miles, a territory approximately half the size of the United States.

India is divided into three main areas: 1) the Himalayan region, being the northernmost mountain wall; 2) the Indo-Gangetic plain; and 3) the Deccan plateau, the southern peninsula that lies in the tropics.

The temperature in the north ranges from a mean of 40° F in January to 115° F in May. The cool winter season, from November through February, is followed by a hot summer period from March to June and a humid, rainy season from July to October. The plains are hot throughout most of the year. The temperature of the Deccan plateau is also hot and fairly constant, but somewhat lower and less humid than the Indo-Gangetic plain. Tropical monsoon climate prevails in most of the country. The average rainfall is 42 inches.

THE POPULATION OF INDIA

India has a population of 438,000,000 people. About 82 percent of these live in villages, while only 18 percent live in cities and towns. Agriculture is the means of livelihood for 70 percent of the population. India has been the melting pot of races since ancient days. If one keeps in mind that there cannot be any rigid separation of types, as there has been considerable overlapping, it can be said that the racial elements consist of the following: 1) Negrito, nearly extinct in India, with only a small group still surviving in the Andamans; 2) proto-Australoid—related to the Australians and Europeans—surviving in a good many aboriginal tribes of present-day India, though more or less mingled with other people; 3) the Mongoloid group, found in the mountainous zones of north and northeast India; 4) a Mediterranean group; 5) Western Brachycephales who apparently have evolved in the Central Asian mountains and have spread over the greater part of India; and 6) Nordics, who are the Aryan-speaking group responsible for the Hindu civilization. The Nordic elements are strong on the northwest frontier, are present in mixed form in northern and central India, and are found in the higher castes spread over this country.

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SOCIAL PATTERN

Since India is a vast and ancient land, the customs and habits of the people differ widely. Many languages are spoken, the dress of the people varies widely, and the food and ways of cooking are different in every section. This has some bearing on the geographic distribution of some diseases. There are 200 villages for each town and few large cities. Medical aid to the villages is meager. All epidemiologic data, particularly for a rare condition such as skin cancer, are available only from the few large cities.

Investigation and treatment of all types of cancer are done on a large scale at Tata Memorial Hospital for Cancer in Bombay. Skin cancer is rare in India and thus there are few published studies giving detailed figures on different types of lesions. The low incidence of cancer of the skin is emphasized by Dr. V. R. Khanolkar, who reported skin cancer in

TABLE 1.—Distribution of basal-cell carcinomas in Indians and Europeans by site

	Indians				Europeans			
	Male	Female	Total	Percent	Male	Female	Total	Percent
Cheek	23	13	36	24. 3	2	3	5	10
Lip	2	—	2	1. 3	1	1	2	4
Chin	—	1	1	0. 6	1	—	1	2
Temple	6	1	7	4. 7	5	—	5	10
Scalp	12	—	12	8. 1	2	—	2	4
Forehead	7	6	13	8. 8	2	3	5	10
Eyelid	19	5	24	16. 2	6	—	6	12
Nose	15	3	18	19. 0	13	5	18	36
Ear	5	1	6	4. 1	1	1	2	4
Abdominal wall	1	1	2	1. 4	—	—	—	—
Neck	3	—	3	2. 1	1	—	1	2
Back and shoulder	3	2	5	3. 4	—	—	—	—
Forearm	1	—	1	0. 6	1	—	1	2
Chest	1	2	3	2. 1	1	—	1	2
Leg	3	—	3	2. 1	1	—	1	2
Vulva	—	1	1	0. 6	—	—	—	—
Anal region	1	—	1	0. 6	—	—	—	—

Indians as 1.4 percent of all carcinomas, while among Europeans residing in India, it was 18.4 percent. Squamous-cell carcinoma was more common than basal-cell carcinoma among the Indian population.

Dr. Sirsat, pathologist at the Tata Memorial Hospital, has analyzed 200 cases of basal-cell carcinoma during the years 1941–55. Of these 200 cases, 148 occurred in Indians and 52 in Europeans. Since the European population makes up only 0.6 percent of the total population in Bombay, it can be seen (table 1) that the incidence of basal-cell carcinoma among Europeans is much higher than among Indians. The maximum age incidence in both groups was between 41 and 60 years. Among Indians, the common sites were cheek, eyelids, and nose, while in Europeans the nose was the site of predilection.

HISTOPATHOLOGY OF BASAL-CELL CARCINOMA

Two histologic types of basal-cell carcinoma were seen in the material studied by Dr. Sirsat.

A. *Reticular pattern.*—The tumor consisted of compact masses of darkly stained cells with the peripheral cells resembling the basal cells of the epidermis. These were spindle shaped, with relatively large vesicular nuclei and scanty cytoplasm. The cells in the center were ovoid and of uniform size. The cords of tumor cells were separated by strands of connective tissue infiltrated by mononuclear cells.

B. *Adenoid cystic pattern.*—The microscopic appearance of this type was characterized by acinar spaces lined by cuboidal columnar cells. The laminae contained basophilic secretion. In areas, finger-like projections dipped into the surrounding stroma from the alveoli.

Table 2 shows the distribution of the 2 varieties of basal-cell carcinoma among different communities in Bombay and compares it with that among the Europeans. The reticular type was more common among Europeans (46 of 52 as against 5 adenoid cystic types). This is a significant difference when one considers that Europeans make up only 0.6 percent of the population of Bombay. Adenoid cystic epithelioma, on the other hand, was frequently seen among Hindus.

TABLE 2.—Distibution of basal-cell carcinomas among races by histologic type

	Hindus	Parsees	Muslims	Indian Christians	Others	Europeans
Population percentage in Bombay	69	4	17	5	4.4	0. 6
Reticular basal-cell carcinoma	46	19	13	8	4	46
Adenoid cystic epithelioma	41	5	3	2	0	5
Intermediate basal-cell carcinoma	3	0	3	2	0	1
Total	90	24	19	12	4	52

PIGMENTATION AND SUNLIGHT EXPOSURE

In India vitiligo, or leukoderma, has been on the increase. There are a large number of individuals with depigmented areas of more than 20 to 30 years' duration on the exposed parts of their body, yet there are no reports in the literature of cancer developing on the vitiliginous areas.

The work of Cipollaro, Elliot, and Welton indicates that it is not the intensity of each single exposure, but rather the total sunshine hours to which the skin is exposed that is the important factor in the production of skin cancer. Most parts of India have as many sunshine hours as the southern parts of the United States. Professor S. K. Mitra's work on the atmosphere has established that ozone absorbs the ultraviolet radiation of the solar spectrum. The maximum concentration of ozone in the atmosphere is located at an altitude of 25 to 30 km. The total ozone content is quite small, 0.1 to 0.2 cm when reduced to standard temperature and pressure, but it suffices to absorb the solar wavelengths from 2900 A to 2100 A. Heavy clouds may absorb or reflect upward much of the ultraviolet radiation. The comparative study of daily ozone fluctuation in different parts of India and at different altitudes is now being undertaken.

The total radiation received from the sun was supposed to be constant. With recent rocket experiments, it has been found, however, that the total radiation given out by the sun varies widely. In meterological terms, this is measured as sun-spot numbers. Every 11th year there is a peak in sun-spot numbers. Considering the sun-spot numbers from 1947, one finds that in 1947 the number was 151.5, while in 1954 it was at its lowest (4.4) and in 1957 there was an unusual high peak in the sun's energy (190.2). The next low figure is expected to occur in 1964. It would be interesting to note whether the incidence of skin cancer also varies in a direct or approximately direct relation to the sun's total energy output. Of course, other factors in the production of skin cancer will have to be considered. Chronic irritation, such as prolonged application of heat, physical friction, and chemical irritation, alone or in combined form, may be a stimulus for the production of cancerous change in the skin or may produce precancerous lesions. In India, the effects of such stimuli have been studied. They differ in various areas according to the habits, customs and particular type of dress of the people.

KANGRI CANCER

In 1900 E. H. Nave first described skin cancer caused by kangri burn in Kashmir (the northernmost part of India, which includes parts of the Himalayan ranges).

Kangri is an earthenware pot covered with a network of wicker work, with a jute thread handle. Burning charcoal or dried leaves are placed into this pot. The wood charcoal is obtained from various sources—willow, witch hazel, chenar leaves, and sometimes pine. Poor Kashmiris tie this pot to their abdomen to keep themselves warm. The heat is obtained from the open mouth and from the walls of the pot. These people wear only one long shirt, and the kangri hanging inside the shirt holds the warmth for longer periods. When these people sit, the kangri pot is kept on the ground between their thighs.

The temperature to which the skin is exposed ranges from 150 to 200° F. Many times this causes burns, and usually scars are present on the abdomen. Many persons use this method of warming themselves from childhood to old age during the long winter months. Women, due to their domestic occupation, do not use kangri continuously for as long periods as the men do.

In the areas in actual contact with heat rays, every stage of chronic dermatitis may be seen—from redness with or without desquamation, to thickened patches, warty induration, or even horny outgrowths. The skin of the abdomen and thighs often is dry and horny. Red papular and squamous lesions are seen on the chest, thighs, and abdomen. Usually epitheliomas arise from the scars of old burns. Tumors appear as single or multiple lesions and are usually confined to the flexor aspects of the body. In the early stages, warty or keratinous thickening is present without erosion, but more commonly there is ulceration and in some instances a fungating mass of cancerous tissue. Curiously enough, the epitheliomas are not solely limited to the parts of the body exposed to heat, but are also occasionally seen on the extensor surfaces and the back. Kangri-burn cancer is common in males, and the average age at onset is 55. It is rare before the age of 40 years and only 6 to 7 percent of the cases are encountered after 70. Metastasis to lymph nodes in the inguinal area is common. Microscopically the kangri-burn cancers are squamous-cell carcinomas.

During a 50-year period from 1890 to 1940, 3,064 cases of kangri-burn cancer have been treated at the Mission Hospital in Kashmir. At the same hospital an average of 45 cases of kangri-burn cancer were seen a year as compared to 10 cases a year of all other types of cancer.

It has been suggested that long, continuous exposure to heat is primarily responsible for kangri-burn cancer. Passing reference has been made to the products of combustion that come in contact with the body. In recent years, probably due to improvement in the economic condition of the Kashmiris, kangri-burn cancer has been less prevalent.

In 1904, Sutherland described another instance of social custom conducive to malignant growths: the use of a blunt razor for shaving of heads, commonly in vogue among the lower class of Mohammedans of northern India. This constant irritation apparently results in some cases of cancer of the scalp.

DHOTI CANCER

In 1945, Khanolkar and Surya Bai, in an analysis of cancer in Bombay (Maharashtra), Patna (Bihar), and Visakhapatnam (Andhra), described three specific types of tumors that they called dhoti, chutta, and khaini cancer. These lesions are intimately concerned with the habits of the people in certain parts of India.

Of the first 5,000 patients attending the clinic at the Tata Memorial Hospital for Cancer in Bombay, there were 3,003 cases with cancer. Carcinoma of the skin was relatively uncommon, occurring only in 63 patients with the squamous- and basal-cell carcinomas equally represented (table 3).

TABLE 3.—Incidence of carcinoma of the skin in Bombay

	Indians		Non-Indians	
	Males	Females	Males	Females
Total attendance at the clinics of Tata Memorial Hospital for Cancer in Bombay	2, 985	1, 655	203	257
Total No. with carcinoma	1, 946	933	71	53
Number with carcinoma of the skin	36 (1. 85%)*	6 (0. 64%)	15 (21. 13%)	5 (9. 43%)
Number with squamous-cell carcinoma	20	3	6	0
Number with basal-cell carcinoma	16	3	9	5

*The figures in parentheses represent percentages of cutaneous carcinoma relative to total carcinoma.

Cancer of the skin of the trunk is relatively uncommon in whites, but fairly common in Indians. Of 23 cases of squamous-cell carcinomas among Indians, 9 (8 males and 1 female) occurred on the trunk. Of the 9 squamous-cell carcinomas, 2 were located on the loin, 5 in the groin, 1 on the buttock, and 1 on the rump. One of the causes for cancer at these sites has been attributed to irritation and chronic pressure due to wearing a dhoti (loincloth) by the Deccani Maharashtrians. The dhoti exerts constant pressure on the loins, the right groin, the inner surface of the right buttock, and along the midline behind the sacrum. Most wearers work and sleep in the same garment and because of lack of clothes-washing places, clean and wash themselves with a dhoti or a saree on the body. Because of this the sites of the body subjected to the greatest friction by the garment are often soiled with dust and sweat and have the least opportunity for appropriate cleansing.

A detailed study was made of the pressure sites on healthy persons habitually wearing a dhoti. The skin above the iliac crest and in the groin was darker, coarser, and slightly corrugated. The central portion of the altered shagreen-like skin was depigmented and glazed. Histologic appearance of biopsies from these sites suggests a marked disturbance of circulation in the dermis and consequent alteration of metabolism in the overlying epithelial cells. These changes are reminiscent of those described in the skin of mice after application of tar or methylcholanthrene. The histologic changes that result in squamous-cell carcinoma (as seen in human material) need no description except to state that the phenomena of proliferation and the supervention of secondary infection obscure the early changes which supply the basis for development of cancer.

CHUTTA CANCER

In Visakhapatnam and the outlying districts of Andhra Pradesh in the mideastern part of India, carcinoma of the hard palate appears to be fairly common. This type of cancer is considered to be due to irritation from the habit of smoking a local type of cigar, called the chutta, with its burning end inside the mouth. Carcinoma of the hard palate in Visakhapatnam stands high on the list of all cancers. Among 285 cases of cancer of the oral cavity, 105 (36.8%) were cancer of the hard palate. This is in striking contrast to Bombay, where chutta smoking is not practiced, and only 62 (6.2%) cases of cancer of the hard palate were seen in 1,000 cases of oral cancer. Subsequently, D. G. Reddy and V. K. Rao made a further study of cancer of the palate due to chutta smoking. Of 275 cases of cancer of the oral cavity, 124 were situated on the hard palate. The tumor is more common in women.

KHAINI CANCER

Lip cancer comprises only 1.7 percent of all cancer in Bombay, whereas in Patna (Bihar) it makes up more than 12.5 percent of all oral cancers. Inhabitants of Bihar, particularly men, are addicted to the use of a dried tobacco leaf and lime mixture. The mixture is freshly prepared by rubbing these 2 substances on the left palm with the thumb of the right hand. A pinch of this mixture is placed in the groove between the lip and the front teeth and is left there until it is diluted with saliva and swallowed. Squamous-cell carcinoma develops frequently on the lips.

BETEL LEAF AND ACCRA NUT—TOBACCO-CHEWING HABIT

The greater incidence of oral cancer, especially of the cheek in those addicted to betel chewing, has been observed by many workers. This habit is prevalent in India, especially in Madras, Trawancore, Bombay, and Assam. The ingredients used are betel leaves, accra-nut powder, or shavings with lime and catachu, with or without tobacco. Many people are habituated to chewing only the accra nut or tobacco with lime. When no tobacco is used, the mixture is chewed and swallowed; when tobacco is used, the chewed quid is kept in contact with the buccal mucosa for a considerable length of time.

In 1933, Orr (Neyoor Hospital) reported 296 cases of cancer of the cheek and 196 cases of cancer of the lower jaw of a total of 669 cases of oral cancer.

Somervell (1944) reported 52 percent cancer of the lips and cheek and 25 percent cancer of the lower jaw of a total of 4,853 cases of oral cancer.

Veliath (1950) cited 41.1 percent cancer of the buccal mucosa and 13.9 percent cancer of the lip among 158 cases of cancer.

In an attempt to correlate the habits of chewing betel leaf-tobacco and smoking with the presence of a precancerous lesion (leukoplakia), F. S. Mehta *et al.* concluded that leukoplakia is more common in persons with a mixed habit of pan chewing and tobacco smoking (table 4).

TABLE 4.—Relationship of leukoplakia to chewing habits

Habit	Number examined	Leukoplakia	
		Number	Percent
No habit	1, 112	1	0. 0009
Pan chewing	1, 898	80	4. 21
Smoking	1, 129	42	3. 72
Pan and smoking	595	42	7. 06
Total	4, 734	165	3. 48

It is believed that the agent responsible for the malignant changes in the oral cavity is the chemical irritation caused by the alkaloids produced by the action of lime on tobacco and that the betel leaf and accra nut by themselves are harmless. It is the combination of tobacco and lime and the prolonged mastication and retention of the quid that cause the irritation. Recently, Sharma (1958) from Assam has pointed out that the variety of betel nut (accra nut) in Assam (which is used without boiling in water) may contain substances with carcinogenic properties.

CANCER OF THE PENIS

It is a well-known clinical observation that cancer of the penis is almost unknown in Jews and Mohammedans, who are circumcised as a religious duty at an early age. The facts predisposing to penile cancer may be long-continued chemical irritation by smegma, which has a low carcinogenic potency, or irritation caused by a tight foreskin. Samson Handley has recorded penile cancer in Hindu immigrants in the Fiji Islands, but not in the aboriginal Fijians who practice circumcision. Valiath reported 198 cases of penile cancer (15.2% of all carcinomas) in a series of 1,300 malignant growths examined at the Stanley Medical College in Madras. Of 198 cases, 193 were Hindus, 4 Indian Christians, 1 Anglo-Indian, and no Mohammedans. Only 2 cases of carcinoma of the penis in Mohammedans have been reported in India (Sutherland and Mague, 1904–05). The other 2 reports are from Ceylon (Coorey, 1944), but in those cases there also was a history of syphilis. Rajam and Rangiah (1953) reported a 0.25 percent incidence of squamous-cell carcinoma of the penis occurring as a complication or sequel to granuloma venereum in 2,999 patients so diagnosed at the College of Venereology in Madras. Of 92 cases reported by D. Sundersivarao (1950–54), none were in Mohammedans. Table 5, taken from various reports, shows the incidence of carcinoma of the penis.

TABLE 5.—Incidence of penile carcinoma in India

Author	Year	Penile tumors	City	Remarks
Nath and Grewal	1937–38	10% of all malignancies in males	Madras	
Kiniand Subbarao	1937	15.5% of total carcinomas	Visakhapatnam	No Mohammedans
Mahadevan	1947	29.2% of all carcinomas in males	Visakhapatnam Cuttuk, Madras	
Valiath	1953	24.2% of all biopsies obtained from males	Madras	
D. Sundersivarao	1950	18.4% of all malignant tumors of males and 4.9% of all malignancies of both sexes	Visakhapatnam	

MALIGNANT MELANOMA

Reddy *et al.* reported 195 cases of melanoma from 1939–53. This material was collected from Madras and Visakhapatnam. Of 195 cases, 131 (67.2%) were melanomas of the skin. Chaphekar and Purandare (King George Hospital, Bombay) reported 39 cases of melanoma, of which 25 (64.1%) were of the skin. Sirsat (Bombay, 1941–55) reported 31 cases of melanomas of the skin from a total of 60 cases (51.7%). In Reddy's and Sirsat's series, 75 percent occurred between the ages of 30 and 60. The incidence was two and a half times greater in males than in females. Although 40 to 50 percent of the melanomas arose from nevi, they can also occur *de novo.*

Fifty-three percent of Reddy's series, 46.2 percent of Chaphekar's series, and 25 percent of Sirsat's series were on the foot. About 75 percent of melanomas of the skin are seen on the lower extremities. That trauma is an exciting factor in the production of malignant melanoma is supported by this higher incidence on the lower extremity, since nearly all the patients in Reddy's series went barefooted. Similarly, in Sirsat's series, 66 percent of the patients walked barefooted.

VARIATIONS IN SKIN CANCER INCIDENCE WITHIN THE UNITED STATES[1]

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SUMMARY

Published and unpublished American data on skin cancer incidence have been reviewed for further information by anatomical site and histologic type. Some more detailed findings, such as the lesser variability in risk by sex, residence, and geographic region for trunk and lower extremities as opposed to face, head, and neck, and upper extremities, are consistent with other evidence on the role of sunlight as a skin carcinogen. The slopes of curves of age-specific rates appeared to be functions of site as well as of histologic type. Despite the fact that face, head, and neck had the highest risks per unit surface area, this site ranked below upper extremities with respect to relative variability in risk for contrasts by sex, residence, and geographic region. Upper extremities also provided a clear-cut instance of different slopes of age-specific rates for basal- and squamous-cell carcinomas, the basal-cell curve being much flatter. The latter result is consistent with clinical impressions of infrequent occurrence of basal-cell carcinomas on the hands of older persons. Further epidemiological studies of skin cancer might emphasize lesions of the hand and arm.—NCI Monogr. No. 10: 225–243, 1963.

CANCER OF the skin presents formidable obstacles to epidemiologic study in the United States. Unlike most other sites, many skin cancers are diagnosed and treated in physicians' offices, and the apparent diagnostic advantage of accessibility of the lesions can be nullified by the need for comprehensive coverage of many sources of reports outside hospitals that maintain different practices for the recording of data. Records in physicians' offices often may indicate little more than the patient's name and omit such items as age and residence, helpful for identification of individuals to eliminate duplicate case reports and necessary for the calculation of rates specific for age and other demographic factors. Also, many specimens escape microscopic examination and, what is worse, the percentage not so examined will vary among communities and with such host characteristics as age and site of the lesion. Skin cancers are usually not lethal and mortality data cannot be relied on to yield information on differentials in risk by age, sex, race, and other characteristics with the same precision as for other sites with less favorable prognosis.

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Despite these difficulties, certain facts about skin cancer in the United States seem established beyond dispute. These include the low risk among Negroes, the continued rise in incidence rates throughout the lifespan, and the higher rates among persons living in southern States (*1–4*). The liability to skin cancer of workers exposed to oils and paraffins is also well known (*5*).

The skin cancer-latitude relationship can be accepted as reflecting primarily exposure to sunlight, specifically to a band of wavelengths in the ultraviolet spectrum. Several observations support this inference: the excess number of lesions (per unit skin surface) on the face, head, and neck—the anatomy most exposed to the elements; the clinical impressions of its more frequent appearance among farmers, sailors, and cowboys (*6*, *7*); experimental induction by ultraviolet radiation of skin cancer in mice and rats (*8*, *9*).

On several points the evidence is equivocal. For example, the true nature of urban-rural differentials in risk remains to be resolved (*10*). While other physical and chemical agents can cause skin cancer, data on their quantitative importance for man are meager. Little information exists on differentials in sex- and age-specific rates by residence and geographic latitude for the individual sites and histologic types.

Awareness of inaccuracies in the basic data collected by cancer registers and morbidity surveys undoubtedly deterred authors of earlier publications from exploiting at the time the full range of items at their disposal. At this juncture a re-examination of some materials available for United States whites may still be useful to see whether rates specific for site and type yield internally consistent patterns and support or contradict currently held impressions.

MATERIALS

The punch cards for the 10-city and Iowa morbidity surveys of 1947 and 1950 were retabulated in more detail by site and histologic type and additional age-specific and age-adjusted rates computed. The skin cancer cases excluded lesions on the lip and external genitalia and mycosis fungoides. The reports for 4 northern and 4 southern cities sufficed for study of the North-South gradients in risk. The Iowa data were not extensive enough for more systematic examination of urban-rural differences and for investigation of the latter I am indebted to Dr. Paul Gerhardt of the New York State Department of Health for supplying me with special tabulations of skin cancers reported from 1958–60 to the upstate New York Cancer Register. The 8-city tabulations were restricted to whites. The representation of nonwhites in the Iowa and New York series was proportionately small, and the results presented reflect the experience of the white population.

It should be emphasized that the 8-city and Iowa data were obtained by review of records in hospitals and physicians' offices, and that roughly one third were diagnosed clinically without microscopic confirmation. In contrast, the requirement that pathology laboratories send copies of all findings of malignancy to the State Department of Health has been an important element in the operation of the New York Register; this, combined with increased referral of tissues for study in recent years, produced a series with more than 90 percent of cases microscopically confirmed.

Another feature of the New York data should be noted. Skin cancer incidence was computed for 3 population groups—urban, rural areas of metropolitan counties, rural areas of nonmetropolitan counties—to segregate the experience of suburban residents from that of persons living in more rural environments. The lowest age-adjusted rates appeared for rural areas of metropolitan counties: urban, male, 45.6, female, 31.1; rural, metropolitan counties, 33.5, 22.5; rural, nonmetropolitan counties, 42.3, 25.3. Since more than half of the suburban population lives in counties adjacent to New York City, this suggests a possible reporting artifact, that some patients were diagnosed and treated in New York City and not reported to the upstate register. The immediate problem of investigating urban-rural gradients was resolved by a restriction of the contrast to the urban and rural, nonmetropolitan experience.

In none of the series were statements on site and type subject to independent audit and they had to be accepted at face value. Nor is there reason to believe that terminology and classifications were entirely consistent among the several sources and some histologic type reports made in 1947 and 1950 might not agree with current concepts and practice. These considerations indicated the use of rather broad classifications of site and type.

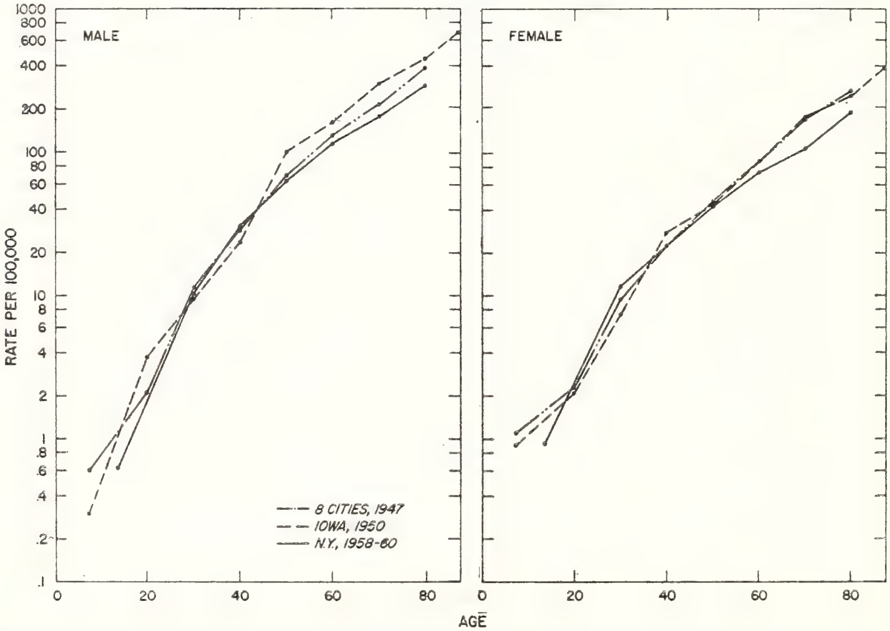
RESULTS

Age and Sex Contrasts

Text-figure 1 presents the age-specific incidence rates for all skin cancers in 8 cities, Iowa, and upstate New York. In the log scale used in this and succeeding figures a constant rate of change with age (exponential growth) would be represented by a straight line. The concordance among the 3 sources was generally good for both sexes up to age 50. The New York rates were lower at older ages, an indication of possible underreporting of clinical diagnoses. Most of the sex disparity in over-all skin cancer incidence was contributed by the older age groups, with only minimal differences appearing up to age 40.

The selected contrasts of age-adjusted incidence summarized in table 1 show that a constant sex ratio in risk does not prevail under all circumstances. The excess male liability appeared more pronounced in the South and among rural residents. The most prominent sex disparity was displayed in the upper extremities, this effect being relatively stronger than that for face, head, and neck, while trunk and lower extremities had sex ratios of risk close to unity. The male predominance was more marked for squamous-cell than for basal-cell carcinomas, and the consolidated data pointed to a small, but definite, female excess risk for melanomas. The Connecticut experience is consistent with the present results on the behavior of the sex ratios by age and anatomical site (*3*).

Text-figure 2 presents age-specific rates by sex for the 4 major anatomical sites (all histologic types combined). The New York and 8-city data agreed that, for each site, incidence increased with age. Face, head, and neck, and upper extremities displayed the sharpest age gradient in risk with approximately equal slopes for these 2 sites among males. The rise with age for trunk and lower extremities was smaller and no important difference in this respect between these 2 sites could be discerned. The figure indicates the reason for the smaller male excess in age-adjusted rates for trunk and lower extremities; the sex differentials in rates to age 40 or 45 were small for all sites, and the more modest rise in rates at older ages for the lower parts of the body allowed the experience at younger ages greater weight in the determination of summary contrasts. About the same results held for each sex, though the female experience for upper extremities constituted a possible exception with both sources pointing to



TEXT-FIGURE 1.—Skin cancer incidence by sex and age, 8 U.S. cities (1947), Iowa (1950), and New York State, exclusive of New York City (1958–60). *Note:* The data for 8 U.S. cities relates to whites only; the proportion of nonwhites in Iowa and upstate New York is small, and the rates shown effectively represent the experience of the white population.

TABLE 1.—Age-adjusted* skin cancer incidence per 100,000 population by sex for selected characteristics: 8 U.S. cities† (1947), Iowa (1950), and New York State, exclusive of New York City (1958–60)

Characteristics	Male			Female			Ratio: Male/female		
	8 Cities†	Iowa	New York	8 Cities†	Iowa	New York	8 Cities†	Iowa	New York
All skin cancers‡	48. 2	61. 4	41. 3	34. 9	35. 3	27. 6	1. 38	1. 74	1. 50
Region									
North	33. 4			25. 4			1. 31		
South	143. 3			88. 0			1. 63		
Residence									
Urban	48. 2	72. 1	45. 6	34. 9	45. 4	31. 1	1. 38	1. 59	1. 47
Rural		52. 1	42. 3§		24. 9	25. 8§		2. 1	1. 64
Site									
Face, head, and neck	39. 4	48. 6	30. 3	28. 2	27. 4	20. 5	1. 40	1. 77	1. 48
Upper extremities	3. 7	5. 3	3. 1	1. 8	2. 1	1. 4	2. 1	2. 5	2. 2
Trunk	2. 8	3. 4	3. 0	2. 9	3. 7	2. 3	0. 97	0. 92	1. 30
Lower extremities	1. 4		1. 0	1. 5		1. 4	0. 93		0. 71
Histologic type									
Melanoma	1. 7	Not computed	2. 3	2. 2	Not computed	2. 6	0. 77		0. 89
Basal- and basosquamous-cell carcinoma	21. 8		26. 8	16. 7		19. 4	1. 31		1. 38
Squamous-cell carcinoma	12. 1		7. 2	6. 7		2. 9	1. 81		2. 5

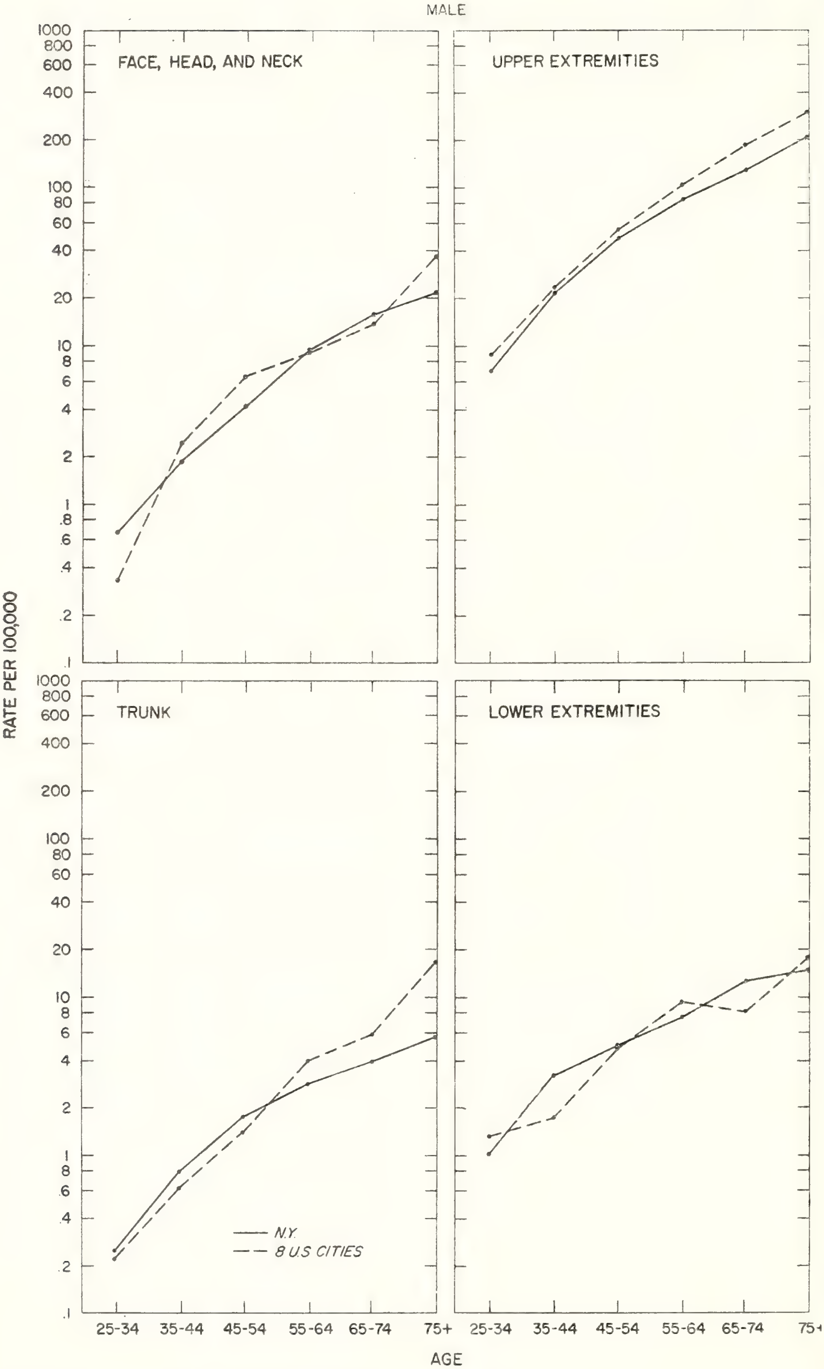
*Age-adjusted to total population of continental United States, 1950.

†Whites only.

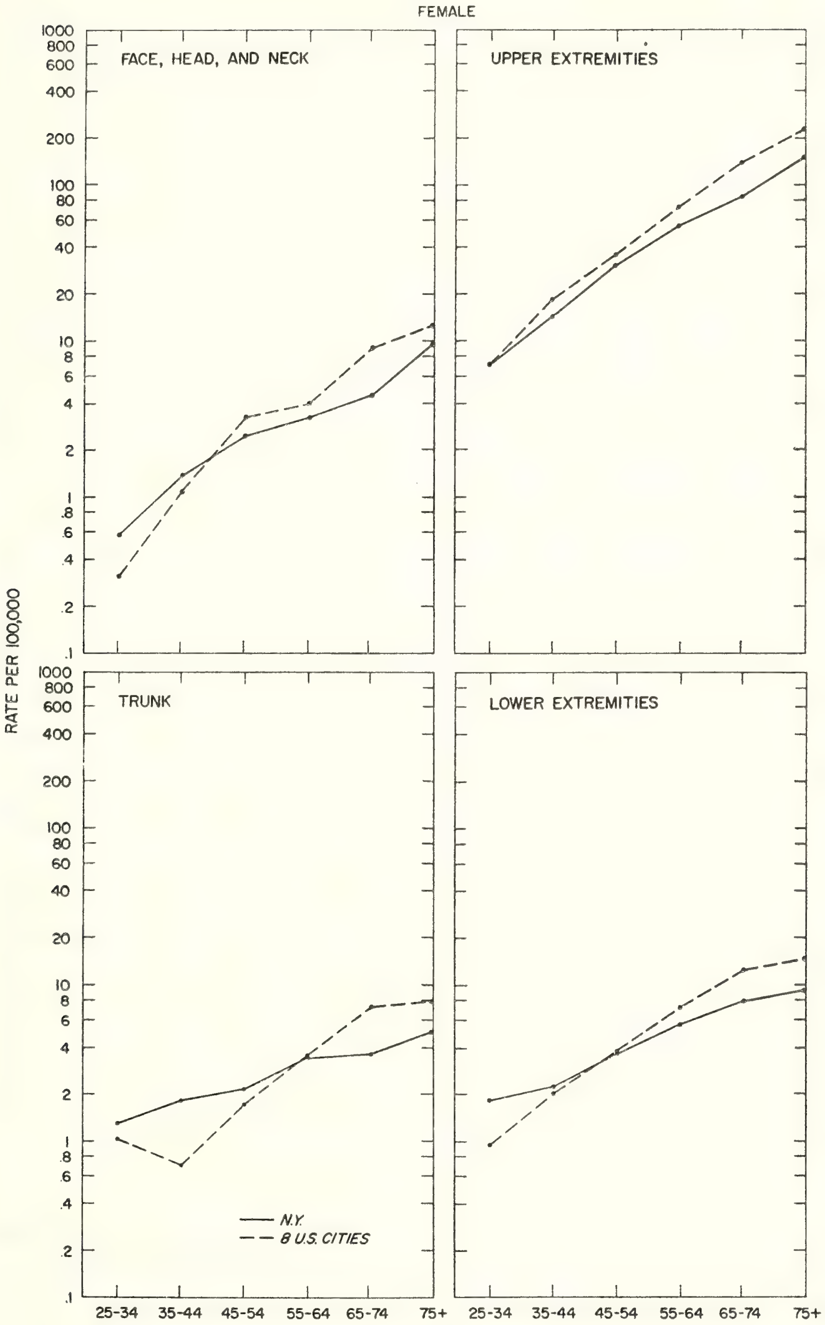
‡Includes newly diagnosed cases of all types and sites, with or without microscopic confirmation.

§Residents of rural areas of nonmetropolitan counties in New York State.

Sources: 8 cities—unpublished tabulations from 10-city cancer morbidity surveys. Iowa—(*4*) supplemented by unpublished tabulations. New York—unpublished tabulations furnished by Bureau of Cancer Control, New York State Department of Health.



Text-figure 2.—Skin cancer incidence by sex, age, and site, 8 U.S. cities (1947) and



New York State, exclusive of New York City (1958–60).

a more shallow gradient in age-specific rates among older women for this site.

The pattern of age-specific rates for the 3 major histologic types is indicated in text-figure 3. The curve for melanomas can readily be seen to have the least progression with age. The range in rates over the lifespan was more compressed for basal- than for squamous-cell carcinomas, and the rise in risk after age 50 or 55 was perceptibly greater for squamous-cell carcinomas. Review of the specific rates singled out a possible sex difference in the age gradient for one site (upper extremities), but none for histologic type.

Age-adjusted rates by sex for site and histologic type combinations appear in table 2, with the corresponding male-female ratios. For both basal- and squamous-cell carcinomas the characteristic excess risk among males came through most strongly for upper extremities and face, head, and neck in that order, this feature being particularly striking for squamous-cell carcinomas. For melanomas the relatively small number of observations and the inconsistent findings preclude fine inferences on differences in sex ratios of risk among anatomical regions.

Table 2.—Age-adjusted* skin cancer incidence per 100,000 population by sex, histologic type, and site: 8 U.S. cities† (1947) and New York State, exclusive of New York City (1958–60)

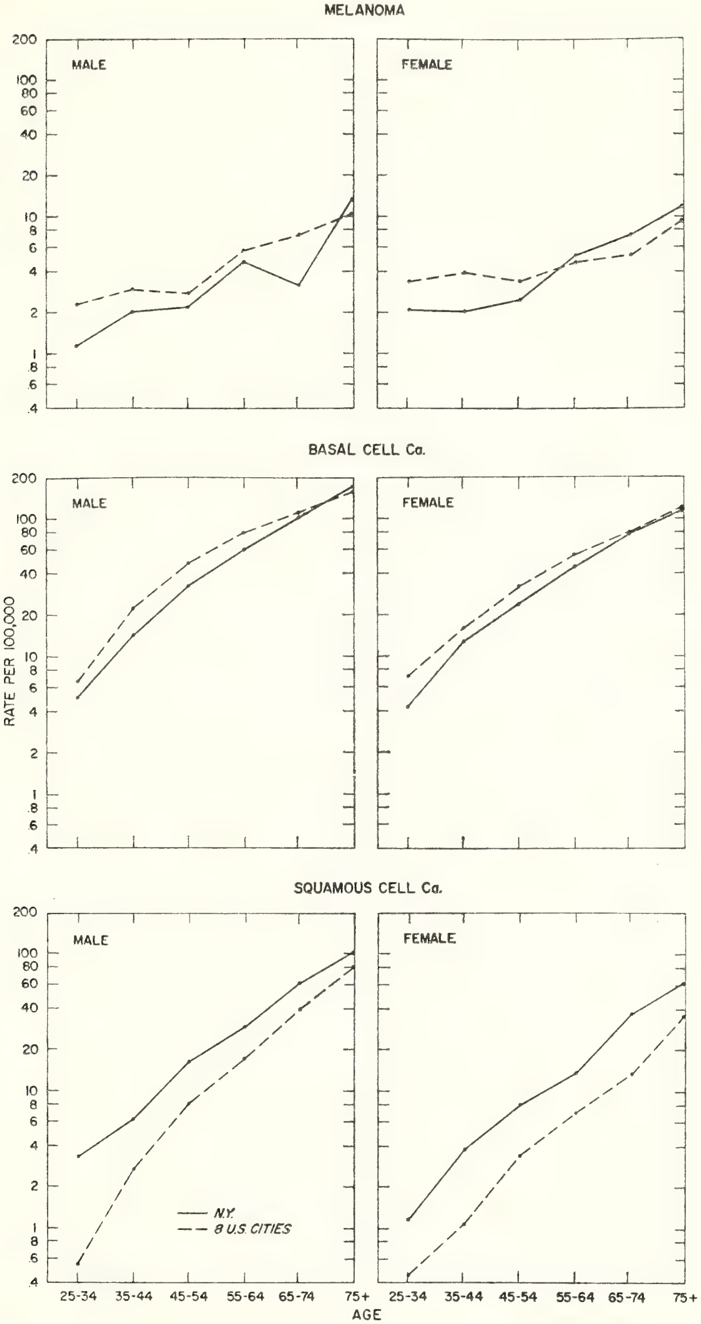
Type and site	Male		Female		Ratio: Male/female	
	8 Cities†	New York	8 Cities†	New York	8 Cities†	New York
Melanoma‡	1.7	2.3	2.1	2.5	0.81	0.92
Face, head, neck, and upper extremities	0.5	0.6	1.0	0.7	0.50	0.86
Trunk and lower extremities	1.0	0.9	1.1	1.2	0.91	0.75
Basal- and basosquamous-cell carcinoma‡	21.8	26.8	16.7	19.4	1.31	1.38
Face, head, and neck	19.6	23.3	14.9	17.0	1.32	1.37
Upper extremities	0.5	1.0	0.3	0.5	1.67	2.0
Trunk	1.1	1.7	1.1	1.3	1.00	1.12
Lower extremities	0.2	0.2	0.2	0.4		
Squamous-cell carcinoma‡	12.1	7.2	6.7	3.3	1.81	2.2
Face, head, and neck	8.2	4.7	4.3	1.9	1.91	2.5
Upper extremities	2.4	1.5	1.0	0.4	2.4	3.8
Trunk	0.8	0.8	0.8	0.9	1.08	0.89
Lower extremities	0.6		0.5			

*Age-adjusted to total population of continental United States, 1950.

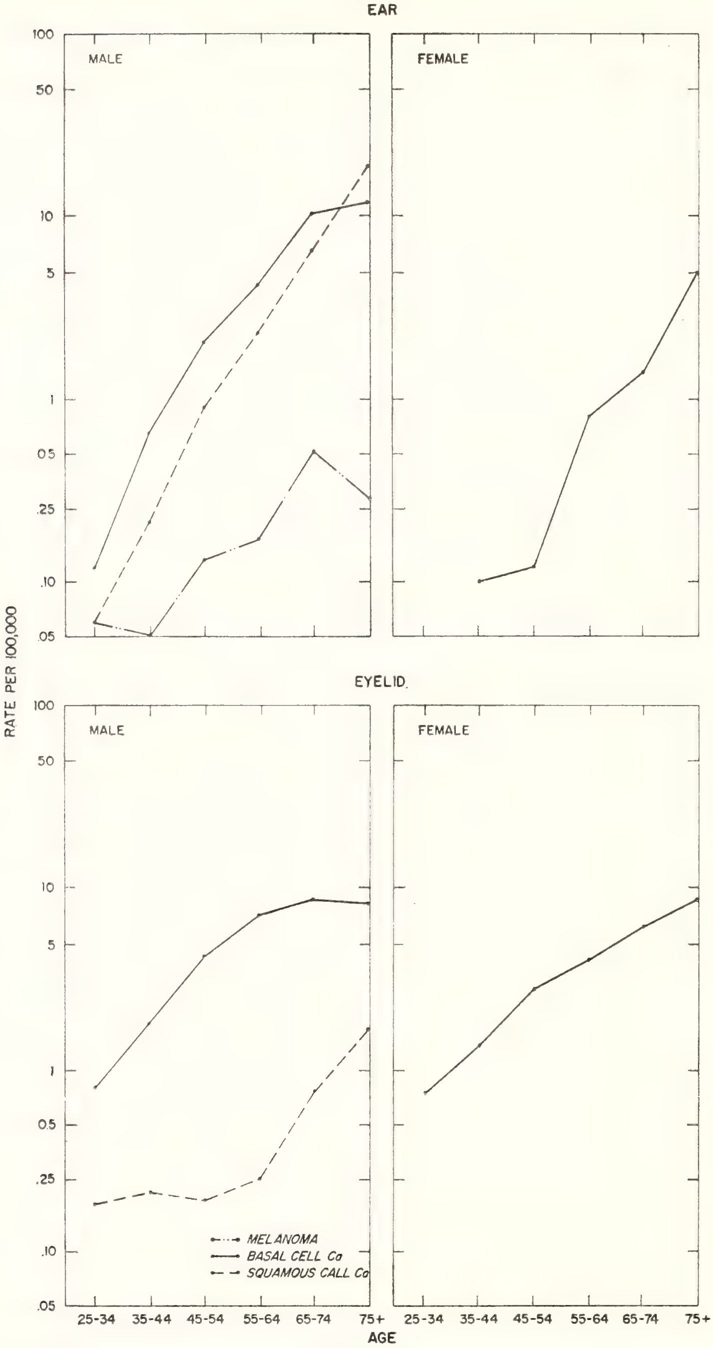
†Whites only.

‡Includes other and unspecified sites.

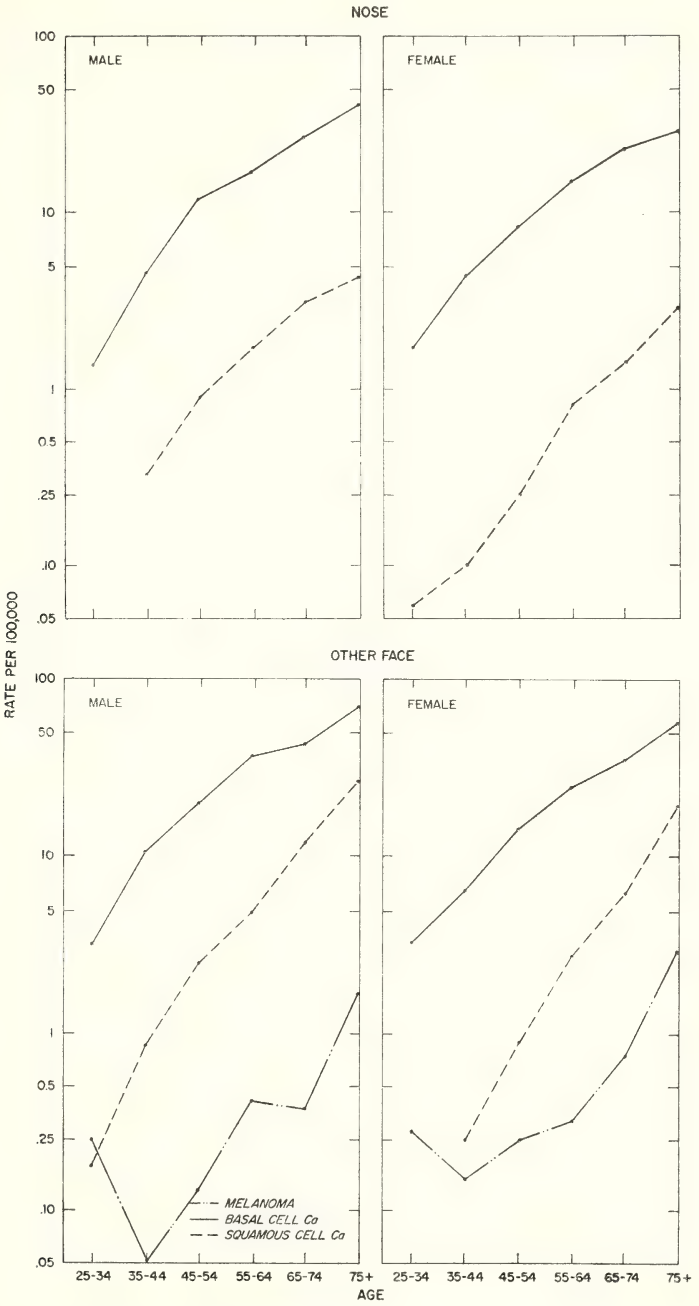
The age-specific rates are studied further in text-figure 4 for combinations of site and histologic type. The individual rates are based on small numbers of observations, and for some sites the data did not warrant presentation for each histologic type, so that any conclusion must be tentative and speculative. The sites for which the slopes of the age curves were roughly parallel for basal- and squamous-cell carcinomas



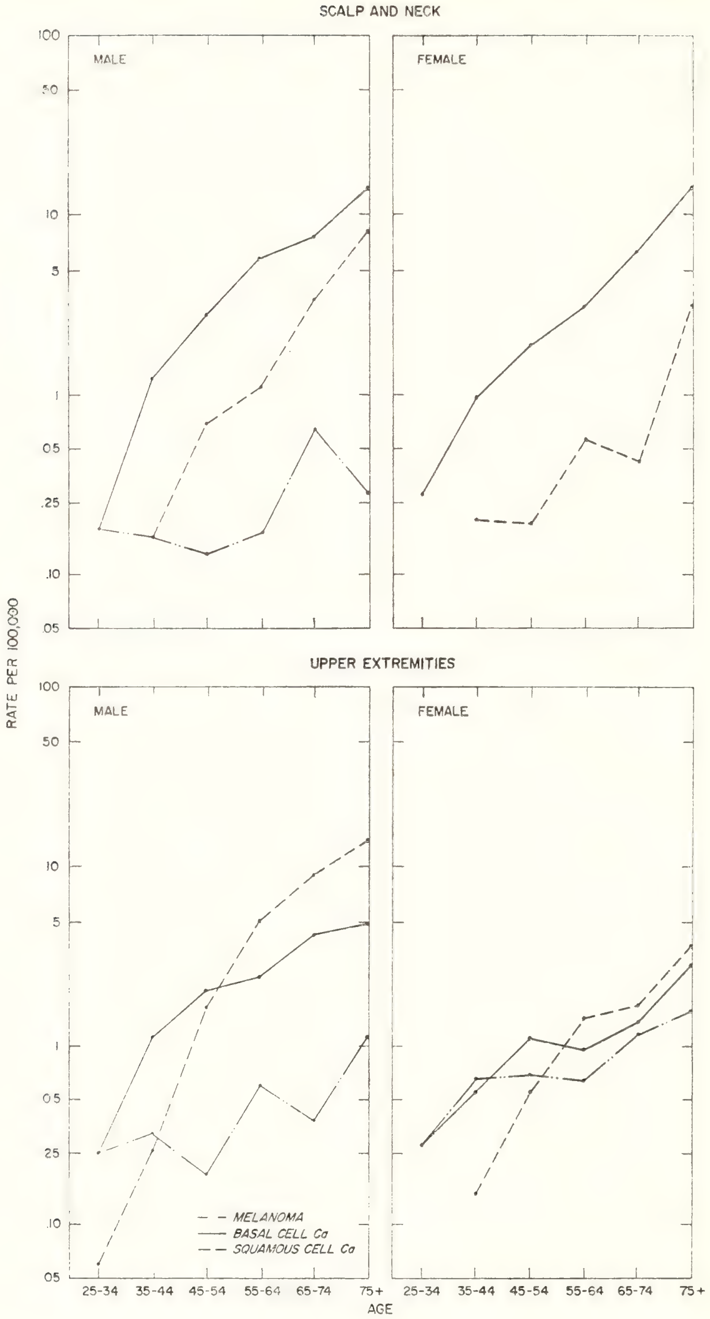
TEXT-FIGURE 3.—Skin cancer incidence by sex, age, and histologic type: 8 U.S. cities (1947) and New York State, exclusive of New York City (1958–60).



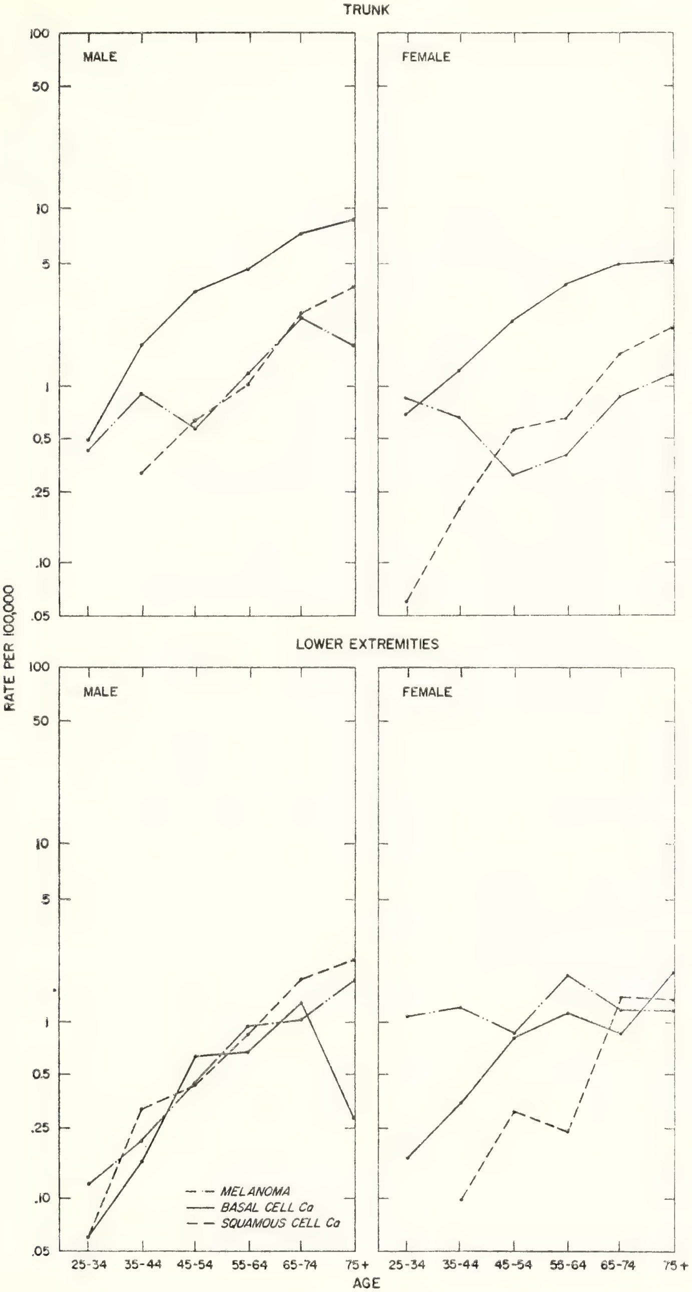
Text-figure 4.—Skin cancer incidence by sex, age, site, and histologic type: New



York State, exclusive of New York City (1958–60)



TEXT-FIGURE 5.—Skin cancer incidence by sex, age, site, and histologic type: New



York State, exclusive of New York City (1958–60).

included most of those on the head with possible exception of eyelid, the trunk, and for males the lower extremities. For upper extremities the slope of the squamous-cell curve appeared much steeper than that for basal cell. Thus, much of the difference in the summary curves for these 2 carcinomas (text-fig. 3) arises from the fact that basal cell is the important component in lesions on the head, where sizable incidence rates appear at younger ages. The increase in risk with age for melanomas on the trunk was not as great as for other types and the shallow slope of the over-all curve for melanomas reflects primarily the experience for this site.

Regional Contrasts

Age-adjusted rates by type and site for 4 northern and 4 southern cities are compared in table 3. While the high skin cancer risks, especially for the face, head, and neck, among whites living in the South have been well documented, the table pinpoints more detailed differences in the behavior of regional gradients. The North-South differential persisted when melanomas were examined separately, albeit on a reduced scale among males. Among females the relative regional standings for melanomas were in line with other types.

TABLE 3.—Age-adjusted* skin cancer incidence per 100,000 population by sex, histologic type, site, and region: 8 U.S. cities† (1947)

Type and site	4 Northern cities		4 Southern cities		Ratio: North/South	
	Male	Female	Male	Female	Male	Female
All skin cancers‡	33.4	25.4	143.3	88.0	0.23	0.29
Melanoma§	1.5	1.6	3.2	5.6	0.47	0.29
Face, head, neck, and upper extremities	0.5	0.7	1.3	2.6	0.38	0.27
Trunk and lower extremities	0.9	0.9	1.2	2.4	0.75	0.38
Basal- and basosquamous-cell carcinoma§	15.7	12.4	61.2	40.6	0.26	0.31
Face, head, and neck	14.3	11.3	55.1	35.6	0.26	0.32
Upper extremities	0.1	0.1	2.3	1.2	0.04	0.08
Trunk	0.9	0.8	2.4	2.2	0.42	0.31
Lower extremities	0.2	0.1	0.2	0.7		
Squamous-cell carcinoma§	8.9	5.3	32.3	14.3	0.28	0.37
Face, head, and neck	5.9	3.5	22.2	9.6	0.27	0.36
Upper extremities	1.5	0.5	8.0	3.0	0.19	0.17
Trunk	0.8	0.8	1.3	0.9	0.70	0.75
Lower extremities	0.6	0.4	0.7	0.7		

*Age-adjusted to total population of continental United States, 1950.

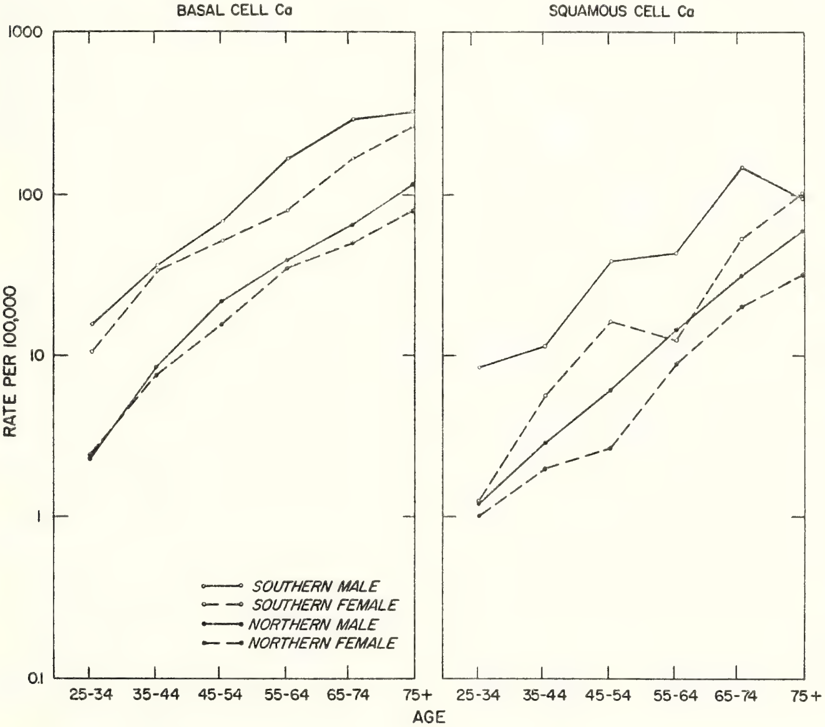
†Whites only.

‡Includes newly diagnosed cases of all types and sites, with or without microscopic confirmation.

§Includes other and unspecified sites.

For basal- and squamous-cell carcinomas the most favorable experience for northern residents was found in upper extremities and face, head, and neck, particularly the former site. The unusually low North-South ratios of risk for basal-cell carcinoma of the upper extremities, a characteristic that carried over in less extreme form to the squamous-cell data, had not been commented on in earlier papers. The general property of lesser variability in risk for trunk and lower extremities vis-a-vis the upper anatomy, noted in preceding tables, prevailed for regional contrasts within each histologic type as well. The female rates for basal-cell carcinoma of the lower extremities (North, 0.1; South, 0.7) may provide an interesting exception to the rule; more data need to be collected before this result can be labeled as fact or artifact. Still, cursory observation would suggest that women's legs are more exposed and this anomaly may reflect a sex difference in exposure permitting a carcinogenic effect from solar radiation to appear in the female data, which is suppressed to minimum threshold levels among southern white males. With this line of reasoning the pattern of regional effects by site for basal- and squamous-cell carcinomas might then be construed as consistent with other evidence on the carcinogenic properties of sunlight.

The age-specific rates for basal- and squamous-cell carcinomas of the face, head, and neck are presented in text-figure 5 for the northern and southern cities. For both types the regional differentials persisted throughout the lifespan.



TEXT-FIGURE 6.—Incidences of skin cancer for face, head, and neck by sex, age, histologic type, and region: 8 U.S. cities (1947).

Urban-Rural Contrasts

The present sources disagree as to the magnitude of urban-rural skin cancer differentials (*10*), so that the New York rates by type and site shown in table 4 cannot be accepted as necessarily characteristic of other findings. The New York data have yielded the most conservative estimates of an over-all excess risk among urban residents, so that they might be regarded as possibly more sensitive in detecting sites and types for which higher rates prevailed in rural populations, whereas other data might show an urban excess in each and every subcategory.

Table 4.—Age-adjusted* skin cancer incidence per 100,000 population by sex, histologic type, site, and urban or rural† residence: New York State, exclusive of New York City (1958–60)

Type and site	Urban		Rural†		Ratio: Urban/rural	
	Male	Female	Male	Female	Male	Female
All skin cancers‡	45. 6	31. 1	42. 3	25. 8	1. 08	1. 21
Melanomas§	2. 5	3. 1	2. 7	2. 3	0. 93	1. 35
Face, head, neck, and upper extremities	0. 7	0. 9	0. 7	0. 8	1. 00	1. 13
Trunk and lower extremities	0. 9	1. 5	0. 9	1. 1	1. 00	1. 36
Basal- and basosquamous-cell carcinoma§	29. 8	21. 9	25. 6	16. 9	1. 16	1. 30
Face, head, and neck	25. 9	18. 9	22. 6	15. 2	1. 15	1. 24
Upper extremities	1. 3	0. 6	0. 6	0. 3	2. 2	2. 0
Trunk	1. 9	1. 6	1. 6	0. 8	1. 22	1. 91
Lower extremities	0. 3	0. 5	0. 2	0. 3		
Squamous-cell carcinoma§	7. 3	3. 2	9. 2	3. 3	0. 79	0. 97
Face, head, and neck	4. 4	2. 1	6. 8	2. 3	0. 65	0. 91
Upper extremities	1. 8	0. 5	1. 4	0. 4	1. 22	1. 25
Trunk and lower extremities	0. 9	0. 6	0. 8	0. 5	1. 12	1. 20

*Age-adjusted to total population of continental United States, 1950.

†Rural areas of nonmetropolitan counties.

‡Includes newly diagnosed cases of all types and sites, with or without microscopic confirmation.

§Includes other and unspecified sites.

The melanoma rates for males displayed less variability than the corresponding basal- and squamous-cell rates. The higher urban female rate for face, head, and upper extremities was not impressive and the data would suggest that an excess liability of urban women to melanomas, if any, has been contributed by lesions on the trunk and legs.

Higher basal-cell rates were observed among urban residents of both sexes in each site group. The high urban-rural ratio for upper extremities, much greater than for face, head, and neck, was provocative despite the small absolute magnitude of the rates. It stood out even more when viewed in conjunction with the squamous-cell results. For the latter type, higher urban rates for upper extremities diverged from the excess in squamous-cell lesions of the face, head, and neck for rural males and the absence of an urban-rural differential for females. Review of the urban and rural age-specific rates for face, head, and neck, and upper extremities uncovered nothing to modify the impressions conveyed by the age-adjusted rates.

The squamous-cell results might offer a basis for reconciliation with reports on the predisposition of farmers and sailors to skin cancer of the face, if it could be shown that squamous-cell lesions constituted the bulk of the series reported. There may be some difficiulities, however, in accommodating the observation that higher urban-rural ratios for face, head, and upper extremities were noted for basal-cell than for squamous-cell carcinomas with the thought that the solar-induced carcinomas of the skin are predominately basal cell in type. Two possibilities can be considered in this connection, that farmers' attire more adequately shields the face and arms or that reporting of basal-cell carcinomas is more complete for city residents.

The dissimilar urban-rural relationships for basal- and squamous-cell carcinomas and the unique findings for upper extremities call for further study.

DISCUSSION

Ratios of age-adjusted rates closer to unity for trunk and lower extremities than for face, head, and neck, and upper extremities in contrasts by sex, residence, and geographic region are in line with the other evidence on sunlight as a skin carcinogen. The fact that the slopes of curves of age-specific rates seem as much a function of site as of histologic type would also be consistent with the notion that each site has its own characteristic protection against exposure to the elements.

The sizable North-South gradient for basal-cell carcinomas in particular supports the idea that these lesions are solar-induced. However, the almost equally large regional gradient for squamous-cell lesions would be difficult to reconcile with the belief expressed by some that the latter are attributable primarily to other causes (*11*). While assertions of different etiologies for the 2 types rest on such observations as the greater relative frequency of squamous-cell cancers among Negroes and the preponderance of basal-cell cancers on the face, head, and neck, the regional differential is best interpreted as indicating sunlight an important etiological factor for squamous-cell carcinomas as well. The strongest indication for other carcinogens in squamous-cell tumors from the data for United States whites reviewed here might be the high male-female ratios of risks for this type.

While face, head, and neck had by far the highest incidence when reduced to risk per unit surface area, it ranked second to upper extremities when relative variability in risks for contrasts by sex, residence, and geographic region is considered. These findings for upper extremities persisted when examined by histologic type: Both basal- and squamous-cell carcinomas exhibited very high male-female ratios of risk and the North-South contrast was particularly marked for basal-cell lesions of the upper extremities. Furthermore, upper extremities provided a strong exception to the tendency toward approximately equal slopes in the age curves for basal- and squamous-cell carcinomas found in several sites, the basal-cell curve being much flatter. The latter would be consistent with a clinical impression of infrequent appearance of basal-cell carcinomas on the hand among older people. More attention to the arm and hand seems indicated with emphasis directed to occupation and other environmental exposures and host characteristics that may contribute to the relative immunity of the hand to basal-cell lesions. This would require more detailed information than is now collected by cancer registers.

The somewhat higher melanoma risks among females conform to statements in the clinical literature that it is seen with equal frequency in both sexes (*12, 13*). The rarity of metastasizing melanoma in infancy and childhood and the better prognosis for this disease in adult women have led to speculation on sex-linked etiological factors (*13, 14*), so that sex ratios of risk quite different from those for basal- and squamous-cell carcinomas were not unexpected. Since melanomas tend to be disfiguring, part of the apparent female excess in risk might arise from more women having lesions removed for cosmetic reasons, though no evidence can be advanced to support this conjecture.

Perhaps more surprising is the North-South gradient in risk for melanomas, almost equal to that for the other types and roughly the same alignment in slopes of the age-specific curves for melanomas and the basal- and squamous-cell carcinomas for some sites—trunk and lower extremities in males and possibly upper extremities in females. Whether the sex and geographic region contrasts can be accommodated satisfactorily within the framework of present theories on melanomas deserves attention. One question left to the judgment of others is whether the similarity in the geographic region contrasts between melanomas and carcinomas contributes to or reinforces the argument based on pathological considerations that melanomas arise from junctional rather than intradermal nevi, and hence are epidermogenic and should be categorized as carcinomas (*15*).

Re-examination of these data was motivated in part to see whether site and type ratios might supplement incidence rates in problems arising from deficiencies in coverage of diagnosed cases: This was on the premise that the age-adjusted risks for sites in the trunk and lower extremities and possibly for melanomas might remain more stable over a wide range of conditions. This requirement was only partially satisfied, and such ratios would generally operate to minimize group contrasts in risks. Nevertheless, site ratios may prove helpful in an assessment of the characteristics of reported series of skin cancer cases.

A more sophisticated analysis of the data has not been attempted, because their quality does not warrant it. Advances in the epidemiology of skin cancer in the United States must come from improvements in data collected rather than through resort to ingenious statistical techniques. One way to secure better data might be to provide special skin cancer protocols calling for more precise description and classification in a few existing population-based cancer registers. Meetings with hospital staffs and medical societies to explain the rationale for the reporting scheme in terms of study objectives would be helpful. Perhaps most important of all, subsidy for a special pathological examination service may be required. If the attending physician feels that the expense of microscopic examination would produce no comparable benefit to the patient, it is difficult to argue that individual patients should bear these research costs. The alternative is to ask physicians to send specimens, which in their judgment would not indicate normal referral for microscopic examination, to a designated study center.

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WORLD INCIDENCE AND DISTRIBUTION OF SKIN CANCER[1]

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SUMMARY

The age-adjusted death rates for skin cancer in 24 countries in 1950–59 were computed. The rates were high in Australia, New Zealand, South Africa, and Ireland, but low in nonwhites of the United States and in Japan. The death rates per age groups in some countries showed the characteristic trend of falling in the older age groups and rising in the younger. It was also found that the geographical distribution of skin cancer stands in negative correlation to that of stomach cancer. In Japan, the frequency of skin cancer is high in the southern prefectures, and among males the frequency is positively correlated with the mean annual amount of sunshine in hours. Computation of age-adjusted incidence rate to skin cancer, in regions where incidence rates for cancers are published, showed that the rate is particularly high in New Zealand.—NCI Monogr. No. 10: 245–255, 1963.

SINCE THE fatality rate of skin cancer is perceptibly lower than that of many other cancers, probably it would not be helpful to rely on mortality for the purpose of studying its geographical distribution. But as the data on cancer morbidity are available only in a few regions of the world, we will first study the death rate for skin cancer in as many countries as possible.

The age-adjusted death rates for 1958–59 were computed from the data furnished us by the courtesy of central statistical administration of 22 countries. The biennial age-adjusted death rates in 1950–57 were computed with the data published by the World Health Organization (*1–8*). The standard population was the total population of 46 countries in 1950. The population in the United States was divided into whites and nonwhites (table 1).

In 1958–59, as shown in text-figure 1, the age-adjusted death rates among males were higher in Australia and South Africa (whites only) than in any other country. Ireland and New Zealand were next, followed by the United States (whites), Switzerland, Scotland, France, Finland, Denmark, Italy, Norway, Canada, Austria, Netherlands, Sweden,

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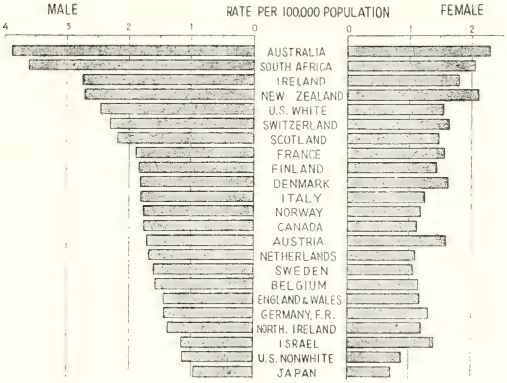
TABLE 1.—Age-adjusted death rates for cancer of skin in 24 countries, for 1950–51, 1952–53, 1954–55, 1956–57, and 1958–59, and sex ratios in 1958–59*

	Male					Female					Sex ratio in 1958–59
	1950–51	1952–53	1954–55	1956–57	1958–59	1950–51	1952–53	1954–55	1956–57	1958–59	
Union of South Africa	4. 18	4. 17	4. 66	3. 75	3. 61(2)	1. 99	2. 73	2. 47	2. 96	2. 05(3)	176
Canada	1. 56	1. 70	1. 70	1. 80	1. 77(13)	1. 12	1. 09	1. 10	1. 17	1. 11(19)	160
Chile	0. 73	0. 66	0. 93	1. 08	—	0. 83	0. 57	0. 73	0. 71	—	—
United States, white	2. 46	2. 46	2. 47	2. 45	2. 46(5)	1. 60	1. 58	1. 59	1. 59	1. 55(9)	159
United States, nonwhite	0. 87	1. 00	1. 13	1. 07	1. 15(22)	0. 92	0. 64	0. 84	0. 85	0. 86(22)	133
Israel	1. 02	1. 15	1. 71	1. 68	1. 16(21)	1. 13	1. 42	1. 22	1. 22	1. 38(12)	84
Japan	0. 88	0. 94	0. 92	0. 94	0. 97(23)	0. 66	0. 69	0. 68	0. 72	0. 70(23)	138
Germany, Fed. Repub.	1. 43	1. 36	1. 34	1. 49	1. 45(19)	1. 50	1. 33	1. 42	1. 45	1. 29(13)	112
Austria	1. 80	1. 43	1. 58	1. 88	1. 72(14)	1. 30	1. 55	1. 32	1. 33	1. 59(7)	108
Belgium	—	—	1. 53	1. 42	1. 57(17)	—	—	1. 31	1. 20	1. 14(18)	138
Denmark	1. 49	1. 79	1. 84	2. 12	1. 82(10)	1. 04	1. 28	1. 61	1. 54	1. 63(6)	112
Finland	1. 29	1. 30	1. 44	2. 00	1. 84(9)	1. 41	1. 14	1. 23	1. 61	1. 44(11)	128
France	2. 31	2. 05	1. 85	1. 78	1. 89(8)	1. 68	1. 66	1. 53	1. 52	1. 57(8)	121
Ireland	3. 16	2. 72	2. 48	2. 96	2. 75(3)	1. 60	1. 95	2. 07	2. 09	1. 79(4)	154
Italy	2. 09	2. 08	1. 85	1. 76	1. 82(11)	1. 38	1. 34	1. 26	1. 32	1. 24(14)	146
Norway	1. 28	1. 47	1. 76	1. 83	1. 77(12)	1. 18	1. 33	1. 56	1. 49	1. 18(16)	150
Netherlands	1. 59	1. 55	1. 34	1. 52	1. 68(15)	1. 11	1. 22	1. 39	1. 30	1. 09(20)	154
Portugal	—	—	—	1. 24	—	—	—	—	1. 10	—	—
England and Wales	1. 90	1. 72	1. 68	1. 62	1. 45(18)	1. 23	1. 28	1. 24	1. 21	1. 16(17)	125
Scotland	1. 64	2. 03	1. 71	1. 91	2. 18(7)	1. 42	1. 15	1. 14	1. 31	1. 47(10)	148
Northern Ireland	1. 53	1. 27	1. 36	1. 64	1. 38(20)	1. 14	1. 39	1. 63	1. 38	1. 18(15)	117
Sweden	0. 65	1. 22	1. 34	1. 44	1. 60(16)	0. 51	0. 89	0. 98	1. 03	1. 05(21)	153
Switzerland	1. 79	2. 31	2. 05	2. 07	2. 32(6)	1. 53	1. 55	1. 89	1. 77	1. 64(5)	141
Australia	3. 57	3. 48	3. 39	4. 09	3. 88(1)	2. 04	2. 12	2. 35	2. 23	2. 30(1)	169
New Zealand	3. 04	2. 84	3. 35	3. 14	2. 71(4)	1. 55	2. 03	1. 76	1. 74	2. 11(2)	128

*Rates per 100,000 population. Standard population: 46 countries in 1950. The figures in parentheses show rank order.



Text-figure 1.—Age-adjusted death rates for skin cancer in 22 countries for 1958–59 (standard population: 46 countries in 1950).

Belgium, England and Wales, West Germany, Northern Ireland, Israel, the United States (nonwhites), and Japan. The rate in Australia, the highest, rose to 3.88 per 100,000 or just fourfold that in Japan.

Among females, the list is similarly topped by Australia, New Zealand, South Africa, and Ireland, followed by Switzerland, Denmark, Austria, France, the United States (whites), Scotland, Finland, Israel, West Germany, Italy, Northern Ireland, Norway, England and Wales, Belgium, Canada, Netherlands, and Sweden—Japan and the United States (nonwhites) being last. The rate in Australia was the highest, 2.30, or more than triple that in Japan.

The trend of change of the age-adjusted death rates from 1950–59 is not uniform by countries (table 1). Since the death rate for skin cancer is so low, the rate in many countries with a small population seems to change from year to year without showing any definite trend. But in some countries, the trend is rather well defined. In the United States (whites), Japan, and Canada, the rate remained generally constant through these years, both among males and females, while it continued to rise in Sweden and was lower in England and Wales.

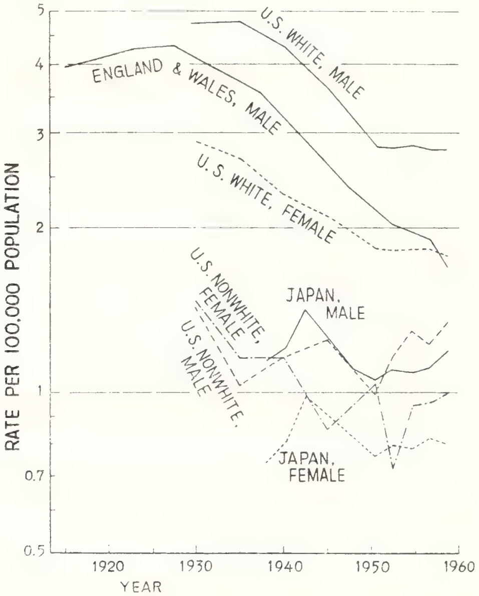
The age-adjusted death rate among males in England and Wales can be computed for the years since 1911 by the data published by Stocks (*9*), in the United States, since 1930, by the reports from the Public Health Service (*10*), and in Japan since 1937. In England and Wales, the rate rose to a peak in 1926–30, but is steadily declining since. In the United States (whites), the rate steadily tended downward until 1945, but since then for unknown causes has ceased to drop, and in recent years the rate among females is higher than among males in England and Wales. In Japan, the peak was reached in 1942–43 but remained constant for 10 years as stated previously (text-fig. 2).

The sex ratio of the age-adjusted death rate for 1958–59 is the highest in South Africa (176), next in Australia (169). Many countries have a ratio of around 150, *i.e.*, Canada, United States (whites), Netherlands, Ireland, Sweden, Scotland, and Italy. Israel alone shows a ratio below

100 (84), *i.e.*, the rate here is higher among females. The difference of the sex ratio by countries is smaller than for cancers of many other sites (table 1).

The death rates for skin cancer per age groups in 22 countries in 1958–59 were published in our report (*11*) on cancer deaths. The following points are specially noteworthy:

Among males, the rate in the age groups 50–54 and below is highest in Australia, but at ages 55–60 to 75–79 South Africa shows the highest rate and in the age group of not less than 80, Ireland, Belgium, and France show particularly high rates. France and Belgium are countries with rather low rates in the younger age groups. In the United States (nonwhites), Israel, Japan, Norway, Sweden, and Finland, the death rate in the older age groups is lower than in many other countries.



Text-figure 2.—Trend of age-adjusted death rates for skin cancer (standard population: United States total population in 1950).

Among females, the rate in the age group 60–64 is high in Australia and New Zealand, and markedly high in the oldest age group in France. In the younger age groups, the rate is lowest in the United States (nonwhites), next in Japan, and is also particularly low in Sweden, England and Wales. The death rate is high among both sexes in the younger age groups in Australia, New Zealand, and South Africa and low in the older age groups in the United States (nonwhites) and Japan, but in Israel, the rate among females is higher than that among males in the older age groups.

The death rates since 1930 by age groups in the United States are available. Among whites of both sexes, the death rate is rising in the younger age groups, but falling in the older ones. But among nonwhites, the rate shows no uniform trend by sex and age group.

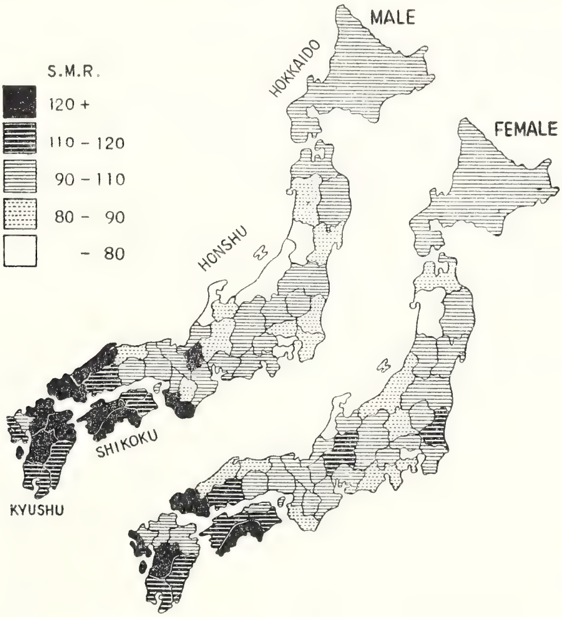
In England and Wales, the death rate among males since 1911–20 has been published by Stocks. Since 1936–40, the death rate is rising in the younger population and declining in the older.

In Japan, the change in the death rate has been available since 1937. Since 1950–51, it has been falling in both sexes in the younger age groups and rising in the older, showing a different trend from that in United States (whites) and England and Wales.

Among males and females the death rates for the biennial periods between 1950 and 1959 inclusive show a rising trend in the younger and a falling trend in the older age groups in West Germany, Australia, and New Zealand (males). It has been rising in the younger and remaining constant in the older age groups in Canada, France, and Italy.

Thus, in most countries, the death rate for skin cancer at older age is approaching that at younger age. Such an evident trend of change has never been observed in the death rates for cancer of any other site in England and Wales, the United States, and Japan, and may be said to be characteristic of skin cancer. Why the death rate for skin cancer should be rising in the younger and falling in the older age groups in some countries is not known.

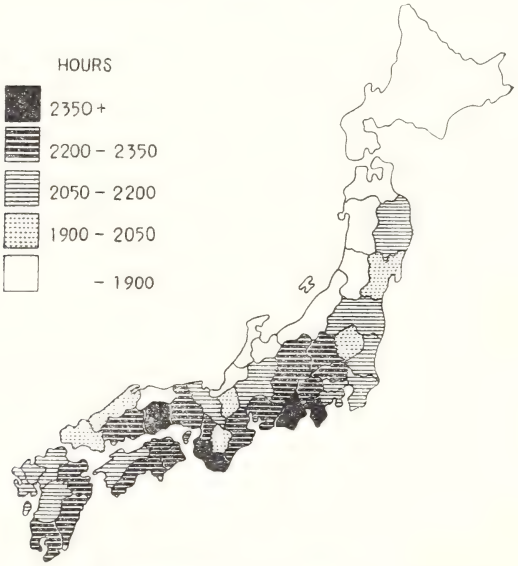
In Japan, the standardized mortality ratio by prefectures, computed from the sum total deaths from skin cancer in the 13 years from 1947 through 1959 (text-fig. 3), showed that the prefectures with high ratios were congregated in Kyushu and Shikoku, the southwestern 2 of



TEXT-FIGURE 3.—Standardized mortality ratio for skin cancer, by prefecture in Japan for 1947–59.

the 4 major islands of Japan. Many of the prefectures in southwestern Honshu also showed a high death rate for skin cancer. Among males and females the rate was highest in Kochi in southern Shikoku facing the Pacific ocean. In this prefecture, the death rate for diabetes mellitus was also highest.

The mean annual amount of sunshine in hours in 1921 through 1950, as shown in text-figure 4, is large in Western Japan facing the Pacific and small in the areas facing the Japan Sea.



Text-figure 4.—Mean annual amount of sunshine in hours, in Japan for 1921–50.

The mean amount of sunshine in the different prefectures in Japan was positively correlated with the standardized skin cancer mortality ratio by prefecture, at the significant correlation coefficient of + 0.440 among males, and at the perceptible but subsignificant coefficient of + 0.261 among females.

Of the 191 cases of skin cancer on which we could obtain information from the outstanding hospitals in all parts of Japan, we found that in

Table 2.—Anamnesis of skin cancer cases in northern and southern Japan

Anamnesis	Northern Japan		Southern Japan	
	Number	Percent	Number	Percent
Burns	15	18. 8	10	9. 0
Injuries	17	21. 3	20	18. 0
Nevus	3	3. 8	17	15. 3
Others	16	20. 0	25	22. 5
Without anamnesis	20	25. 0	29	26. 1
Unknown	9	11. 3	10	9. 0
Total	80	100. 0	111	100. 0

the northern half, above 36 degrees latitude, skin cancer secondary to accidental burn was far more frequent (18.8%) than that secondary to nevus (3.8%), but in the southern half, below 36 degrees latitude, the relation was reversed (9.0% and 15.3%) (table 2). When cases of skin cancer secondary to accidental burns are excluded, the difference between the death rate in north and south would be still larger. Apparently burns are more apt to occur in the north than in the south. It is said that in northern Honshu, charcoal makers are prone to suffer from burns, which in some cases eventually become skin cancer.

Skin cancer on the lower limbs and scalp was more common in the northern half, but occurred more frequently on the face, upper limbs, and neck in the cases reported in the southern half of Japan (table 3).

TABLE 3.—Skin cancer by site in northern and southern Japan

Site	Northern Japan		Southern Japan	
	Number	Percent	Number	Percent
Scalp	19	23. 8	16	14. 4
Face	23	28. 8	41	36. 9
Neck	1	1. 3	5	4. 5
Upper limbs	—	—	13	11. 7
Trunk	10	12. 5	13	11. 7
Lower limbs	27	33. 8	23	20. 7
Total	80	100. 0	111	100. 0

We then studied the geographical correlation of skin cancer with cancers of other sites, using the age-adjusted death rates of 24 countries for the respective cancers. The method of computation may be criticized, but we have arrived at the results, stated later, suitable at least for immediate reference (table 4). Among males, a significant positive correlation was found between the rate for skin cancer and intestinal cancer for 1958–59 and 1956–57: Where the death rate for skin cancer was high, that for intestinal cancer was also high. In 1952–53 and 1954–55 the correlation coefficient was rather high, but not significant. Among females, too, there was a rather close, though not significant, positive correlation between the two. With stomach cancer, skin cancer was found in significant negative correlation among males in 1956–57, and the correlation coefficient was rather high in the other biennial periods also. The negative correlation was apparent among females, the correlation coefficient being large enough to be significant in 1954–55. Among males, skin cancer was significantly positively correlated with prostate cancer in 1954–55 and with cancer of the buccal cavity in 1952–53, while among women it stood in close positive correlation with breast cancer, but significant negative correlation with uterine cancer in 1954–55.

We estimated the prefectural correlation in Japan and found a noticeable negative correlation with stomach cancer. The standardized mortality ratio for stomach cancer by prefectures is high for both sexes in the northern prefectures facing the Japan Sea and low in southwestern Japan,

TABLE 4.—Correlation coefficients between age-adjusted death rates for cancer of skin and those for cancer of selected sites in 24 countries for 1952–53, 1954–55, 1956–57, and 1958–59

	1952–53	1954–55	1956–57	1958–59
Male				
Buccal cavity and pharynx	+0. 47*	+0. 37	+0. 39	+0. 40
Esophagus	−0. 06	−0. 17	−0. 26	−0. 06
Stomach	−0. 39	−0. 40	−0. 40†	−0. 35
Intestine	+0. 35	+0. 39	+0. 47*	+0. 47*
Rectum	+0. 03	−0. 01	+0. 16	+0. 00
Liver and biliary passages	−0. 23	−0. 30	−0. 40	−0. 35
Pancreas	+0. 27	+0. 37	+0. 34	+0. 37
Larynx	—	—	—	+0. 03
Lung	+0. 05	+0. 04	+0. 13	+0. 03
Prostate	+0. 34	+0. 44*	+0. 26	+0. 26
Bladder	—	—	—	+0. 41
Thyroid gland	+0. 05	+0. 07	+0. 07	+0. 15
Leukemia	+0. 16	+0. 33	+0. 40	+0. 27
Female				
Buccal cavity and pharynx	−0. 00	+0. 19	+0. 20	+0. 21
Esophagus	−0. 17	−0. 25	−0. 16	−0. 01
Stomach	−0. 27	−0. 42†	−0. 35	−0. 34
Intestine	+0. 27	+0. 41	+0. 36	+0. 36
Rectum	−0. 06	+0. 08	+0. 15	+0. 09
Liver and biliary passages	−0. 04	−0. 23	−0. 18	−0. 25
Pancreas	+0. 31	+0. 25	+0. 20	+0. 19
Larynx	—	—	—	+0. 03
Lung	+0. 18	−0. 04	+0. 18	+0. 06
Breast	+0. 31	+0. 46*	+0. 43*	+0. 35
Uterus	−0. 35	−0. 44†	−0. 34	−0. 29
Ovary	+0. 16	+0. 15	+0. 12	+0. 26
Bladder	—	—	—	−0. 06
Thyroid gland	+0. 32	+0. 14	+0. 22	+0. 25
Leukemia	+0. 20	+0. 34	+0. 23	+0. 41

*Significant positive coefficient at the level of 5 percent.
†Significant negative coefficient at the level of 5 percent.

apparently a reverse distribution to that of skin cancer. In fact, the correlation coefficient between this prefectural standardized mortality ratio for stomach cancer and that for skin cancer stands at −0.487 among males and −0.488 among females, significantly negative among both sexes. Computation with standardized mortality ratio for stomach cancer in 1951–53 as basis led to a similar result. It is rather interesting that such a tendency of negative correlation should be observed in the distribution of stomach and skin cancers in the countries of the world as well as in the prefectures of Japan.

The fatality rate of skin cancer is so low that its share in the mortality statistics of cancer of all sites is small. But in morbidity statistics of cancers, the ratio of skin cancer is significant in many countries. We have available the results of cancer morbidity surveys in Denmark (*12*, *13*), 10 metropolitan areas in the United States (*14*), Norway (*15*), Finland (*16*), Sweden (*17*), New Zealand (*18*), and Hawaii (*19*). In Japan the results of our survey (*20*), in Miyagi Prefecture are the only available data. The ratio of skin cancer among males is 23.1 percent in New Zealand and 16.7 percent in the United States (whites). Indeed, in these regions, the percentage of skin cancer tops the list. In rural Denmark and Norway, it stands at 12.6 percent, ranking next only to stomach cancer in the former and to stomach and prostate cancers in the latter region. In the United States nonwhites and Japanese in Hawaii it falls to about 2 percent, and and in Miyagi Prefecture, Japan, it is not more than 0.6 percent.

Among females, the ratio of skin cancer is 15.5 percent (ranking second to breast cancer) in new Zealand, 11.8 percent or third to breast and uterine cancer in the United States (whites), and 10.6 percent or next to breast, stomach, and uterine cancers in Norway. In the United States nonwhites, Japanese in Hawaii, and Miyagi Prefecture, Japan, the ratio is particularly low, as among males (table 5).

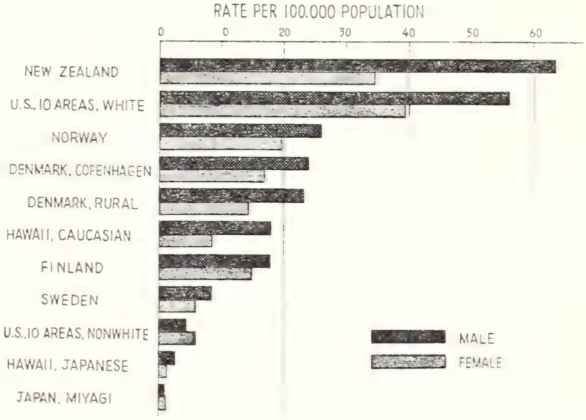
Table 5.—Percentage of newly diagnosed cases of cancer by selected sites

Male	Skin	Stomach	Intestine	Rectum	Lung	Prostate
United States, 10 areas:						
White (1947)	16. 7	9. 9	8. 0	6. 6	9. 4	9. 0
Nonwhite (1947)	2. 0	15. 4	5. 8	5. 0	10. 9	15. 7
Japan, Miyagi (1951–52)	0. 6	53. 3	2. 2	2. 9	2. 5	0. 6
Denmark:						
Copenhagen (1948–52)	8. 5	12. 8	8. 0	8. 7	15. 6	7. 4
Rural (1948–52)	12. 6	22. 2	7. 8	9. 2	4. 3	8. 2
Finland (1954)	6. 9	28. 7	2. 6	2. 0	20. 5	5. 2
Norway (1953–54)	12. 6	23. 7	5. 8	3. 7	5. 1	13. 2
New Zealand (1953–55)	23. 1	8. 4	6. 7	5. 2	11. 9	8. 9
Hawaii:						
Caucasian (1947–54)	9. 9	11. 1	11. 1	4. 6	11. 3	11. 3
Japanese (1947–54)	1. 9	32. 7	6. 8	5. 7	8. 1	2. 0
Sweden (1958)	4. 4	17. 1	8. 4	6. 7	8. 0	16. 7

Female	Skin	Stomach	Intestine	Breast	Uterus	Ovary
United States, 10 areas:						
White (1947)	11. 8	5. 3	8. 4	22. 0	17. 3	4. 6
Nonwhite (1947)	2. 0	6. 5	5. 1	18. 6	34. 3	3. 9
Japan, Miyagi (1951–52)	0. 8	29. 7	2. 6	8. 0	25. 7	1. 3
Denmark:						
Copenhagen (1948–52)	6. 6	8. 9	8. 1	20. 9	19. 3	5. 6
Rural (1948–52)	7. 3	14. 6	8. 2	19. 1	13. 2	5. 0
Finland (1954)	7. 8	22. 9	4. 0	13. 9	13. 9	4. 3
Norway (1953–54)	10. 6	15. 2	5. 6	19. 7	12. 6	5. 4
New Zealand (1953–55)	15. 5	5. 7	10. 4	21. 0	12. 6	4. 1
Hawaii:						
Caucasian (1947–54)	4. 2	5. 3	11. 7	26. 8	20. 4	5. 3
Japanese (1947–54)	1. 1	19. 6	6. 2	11. 0	22. 2	4. 2
Sweden (1958)	2. 8	8. 8	7. 5	24. 0	16. 0	7. 4

We computed the age-adjusted incidence rates for skin cancer in the areas where the data on cancer morbidity were available, and used the total population of the United States in 1950 as standard (table 6, and text-fig. 5).

Among males, the incidence rate was highest in New Zealand, next among whites in 10 areas surveyed in the United States, followed in descending order by Norway, Copenhagen, rural Denmark, Caucasians



Text-figure 5.—Age-adjusted incidence rates for skin cancer (standard population: United States total population in 1950).

Table 6.—Age-adjusted morbidity incidence rates for cancer of skin in selected areas*

Area	Year	Male	Female
United States, 10 areas, white	1947	56. 1	39. 5
United States, 10 areas, nonwhite	1947	4. 5	6. 0
Japan, Miyagi	1951–52	1. 1	1. 3
Denmark, Copenhagen	1948–52	24. 0	16. 9
Denmark, rural	1948–52	23. 2	14. 4
Finland	1954	17. 9	15. 0
Norway	1953–54	26. 0	19. 8
Sweden	1958	8. 6	5. 8
New Zealand	1953–55	63. 5	34. 5
Hawaii, Caucasian	1947–54	18. 0	8. 6
Hawaii, Japanese	1947–54	2. 7	1. 4

*Rates per 100,000 population. Standard population: United States total population in 1950.

in Hawaii, Finland, Sweden, nonwhites in the 10 areas of the United States, Japanese in Hawaii, and Miyagi Prefecture in Japan.

Among females, the order was similar, except that whites in the 10 areas of the United States preceded New Zealand and Finland came next to Copenhagen. The regional difference in the rates of incidence is apparently larger than that in death rates. Of the age-adjusted death rate, the highest among the 22 countries was about three or four times as high as the lowest, but of the incidence rate, the highest-lowest ratio was more than 20 to 1.

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KERATOACANTHOMA[1]

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SUMMARY

The keratoacanthoma is a follicular hyperplasia, a potential squamous carcinoma, at least in the experimental animal. It is normally capable of regressing spontaneously, but may fail to do so if either the response to carcinogenic agents has been sufficiently intense or prolonged, or damage to the connective-tissue stroma or factors unknown inhibit the cyclical influences to which the follicle is normally subjected.—NCI Monogr. No. 10: 257–273, 1963.

THE SLOW and grudging acceptance of the keratoacanthoma as an entity provides a revealing example of the reluctance of our profession to abandon established concepts and cherished definitions. Although it is only in the past 12 years that the keratoacanthoma has gained general recognition as a common and distinctive tumor, many case reports can be found in the literature of the previous 80 years. Some are claimed as successful cures of cancer by a variety of chemotherapeutic procedures; others are discussed skeptically in occasional reviews of self-healing cancer, which most of the writers consider a contradiction in terms. MacCormac and Scarff's paper (*1*) attracted little attention; a few letters of mild protest suggested that this was no more than an already familiar form of early squamous carcinoma.

The clinical and histological portions of the present report are based largely on a detailed study now in progress of 222 cases of keratoacanthoma from the files of the United Cambridge Hospitals and on our earlier experience of these lesions (*2*, *3*). The account of the experimental aspects owes most to the publications of Rous, Cowdry, Ghadially, and Whiteley. In the statistical analyses that follow we have excluded 19 lesions which were in some way atypical. Three patients had 2 lesions. In the age analysis they are included once only, but in the site analysis each lesion is included.

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[2] We wish to express our indebtedness to those clinical colleagues who have allowed us to include their cases; to Dr. G. A. Gresham, Department of Pathology, University of Cambridge, for his interest and cooperation; and to Mr. L. Beard, A.I.B.P., A.R.P.S., Department of Medical Photography, for his illustrations.

INCIDENCE

The keratoacanthoma is a common lesion, but its actual incidence is difficult to assess, partly because the case material referred to departments of surgery, dermatology, and radiotherapeutics may not be comparable, and partly because diagnostic criteria are not uniform. Table 1 illustrates the relative incidence of keratoacanthoma and squamous carcinoma as recorded by authors in several different countries. The differences are, with two exceptions, less striking than the general agreement. The incidence of carcinoma is considerably higher in Sydney, Australia, than in Cambridge, yet the ratio of keratoacanthoma to carcinoma in 2 large series of cases is identical. The diagnosis, as in Beare's (*4*) and Veidenheimer's (*5*) cases was based on combined clinical and histological assessment. Brothers (*6*) and Liban (*7*), relying solely on histological criteria, found carcinoma relatively more frequent. Vilanova's figures (*8*), also based on histological criteria, are more difficult to explain, and a much larger series of cases would be required to support his statement that the keratoacanthoma is rare in Spain. The keratoacanthoma, like the squamous carcinoma, appears to be exceptional in the Negro.

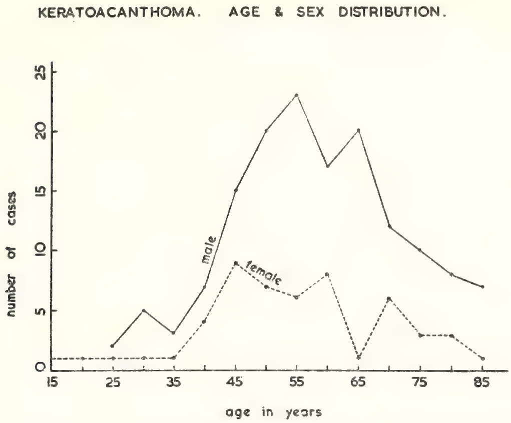
TABLE 1.—Relative incidence of keratoacanthoma and squamous carcinoma

Author	Country	Year	Ratio keratoacanthoma squamous carcinoma
Beare	N. Ireland	1953	1:2
Liban	London	1955	1:11
Belisario	Sydney	1956	1:3
Veidenheimer	W. Canada	1956	1:2.5
Vilanova	Spain	1958	1:17
Brothers	U.S.A.	1960	1:5
Present series	Cambridge, England	1962	1:3

AGE AND SEX

The age incidence of keratoacanthoma in 203 patients is shown in text-figure 1. In both sexes the lesion is most frequently seen between the ages of 45 and 65, but when allowance is made for the age structure of the population-at-risk it is apparent that the actual incidence remains fairly constant after 55. According to the latest available census returns for our area, 24.3 percent of the population are aged 45 to 65, but only 7.3 percent are 70 or over. At all ages males are affected approximately 3 times as often as females. These findings agree generally with those of other investigators. The youngest patient reported (*9*) was 16 months old, and fewer than 3 percent of cases occur below the age of 30.

The age and sex incidence of squamous carcinoma are very different. Our analysis of the clinical records and histology of some 600 cases of squamous carcinoma is still in progress. Table 2 compares the age incidence of 203 of our cases of keratoacanthoma with that of the first 50



Text-figure 1.—Age and sex incidence of keratoacanthoma in 203 patients.

of our cases of squamous carcinoma. The numbers are small but fully confirm our earlier findings (*3*). The incidence of squamous carcinoma increases steadily with age and over 80 percent of the patients are male. If we again make allowance for the age structure of the population, the increase in the incidence of squamous carcinoma with age is further accentuated.

Table 2.—Keratoacanthoma and squamous carcinoma comparative age incidence

	Keratoacanthoma (%)	Squamous carcinoma (%)
Under 39	7	0
40–49	17	6
50–59	28	10
60–69	23	36
70–79	16	30
80–89	9	18

ETIOLOGICAL FACTORS

Environmental

That prolonged exposure to sunlight predisposes to the development of keratoacanthoma is suggested by the parallel variation in incidence of keratoacanthoma and squamous carcinoma in England and Australia, and by the frequency of involvement of exposed skin in the outdoor worker. Occupational exposure to tar and mineral oils (*10*) has also been shown to be associated with an increased incidence of keratoacanthoma. The development of large numbers of lesions in a patient whose psoriasis had been treated with tar has been reported (*11*).

In a proportion of cases the development of the individual keratoacanthoma is apparently initiated by trauma, often trivial—a razor cut, a burn, or a prick with a thorn—which is followed within a week by the appearance of the lesion. The frequent history of recent trauma suggested that an infective agent may be incriminated. Experiences with skin grafting in patients with keratoacanthoma lend weight to this suggestion. In Pillsbury and Beerman's case (*12*) multiple keratoacanthomas developed in the graft. In another case (*13*) multiple lesions developed in donor and recipient sites, and in two other cases (*14, 15*) only in the donor sites. However, the experimental studies of Gillman and his colleagues (*16*) on the epithelial proliferation occurring during the normal healing of Thiersch-graft donor sites offers a possible alternative explanation for these observations.

In our experience the keratoacanthoma rarely arises in a pre-existing lesion, in sharp contrast to squamous carcinoma which, in a high percentage of cases, originates in keratoses, scars, or chronic inflammatory lesions. Although the keratoacanthoma usually develops in skin that is grossly normal, chronic solar degenerative changes are frequent and in one of our own cases, a girl aged 13, and in several reported in the literature (*17–19*) xeroderma pigmentosum was present.

Heredity clearly is important in the multiple self-healing epithelioma of Ferguson-Smith. The individual lesions may be clinically and histologically indistinguishable from keratoacanthoma, but differ in their early age of onset and in their distribution. We will exclude those cases from discussion, as they constitute a special problem, since no genetic factor has been demonstrated either in solitary keratoacanthoma or in the multiple forms occurring in those exposed to occupational hazards.

CLINICAL FEATURES

The clinical features of keratoacanthoma show a remarkable uniformity. A firm hemispherical papule increases rapidly in size to reach a diameter of 1 to 2 cm in 4 or 5 weeks (fig. 1). The lesion may be clinically recognizable after as little as 1 week. After this initial period of rapid growth there is usually no further enlargement. When the typical lesion has reached its maximum size it is a smooth, rounded nodule and its greatest diameter lies somewhat above the base, which it may overhang. It is covered by tense, shining epidermis usually of normal color, but sometimes a bright pink. The center of the lesion is umbilicated and may be covered by a crust that is readily removed and beneath which is a crater filled with horn (fig. 2). Sometimes the horn projects a cm or more above the rim of the crater. The nodule at this stage is of firm, somewhat rubbery consistency, but often it soon becomes softer and flatter. The majority of keratoacantomas begin to undergo spontaneous regression after 6 or 8 weeks. The central plug loosens and soon falls out. The rim flattens progressively until after 10 or 12 weeks only a soft rolled edge remains. This, too, separates to leave a characteristic puckered scar (fig. 3).

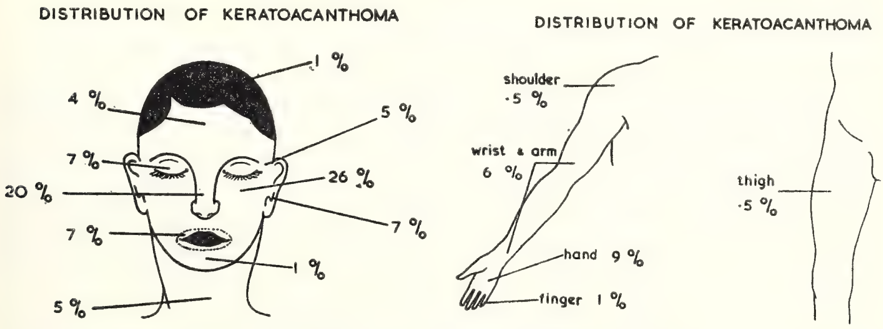
Some 80 percent of lesions probably evolve in this distinctive fashion. The deviations from what we may regard as normal behavior are of special interest, and we are currently engaged on a detailed study (*20*) of those in our own collection, or reported to us by colleagues, which have shown striking aberrations in their clinical behavior. An occasional initial rapid enlargement to achieve a diameter sometimes much in excess of 2 cm in 6 weeks is far more commonly seen in lesions on the forearms, trunk, or in the eyebrow than elsewhere. The few massive lesions reported have been in these sites. Persistence for a year or more is not uncommon, but in most cases the lesions cease to enlarge after 6 or 8 weeks and do not ulcerate. Although lesions in any site may persist, those on the lips, the back of the hands, and forearms are particularly liable to do so.

The liability to recurrence after superficial removal with a curette or scalpel may be dependent on the stage of development of the lesion. In our experience, recurrences have usually followed removal during the growth phase, but they may occur later and have been reported 1 month after apparently complete spontaneous resolution (*4*). The recurrent lesion normally follows the typical benign course if it is allowed to do so. Biopsy usually has no obvious influence on the development of keratoacanthoma, but occasionally may provoke rapid growth even in sites in which large lesions are exceptional (*4*).

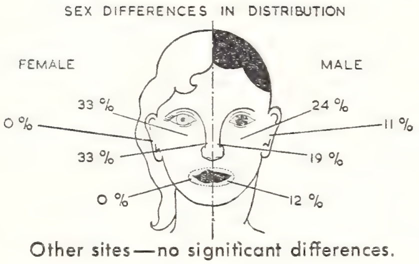
A distinctive variant is the so-called aggregated keratoacanthoma (*21*). Two or more keratoacanthomas develop simultaneously, become confluent, and involute spontaneously. A colleague (*22*) allows us to report on his patient in whom, 11 weeks after the first appearance of a typical keratoacanthoma, which was already showing signs of involution, a cluster of small satellite lesions developed around its periphery, enlarged, and merged with the original lesion.

DISTRIBUTION

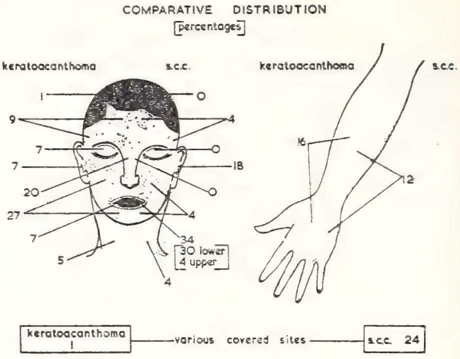
The distribution of keratoacanthoma is shown in text-figures 2 and 3. The majority of lesions occur on exposed skin, and the frequency with



Text-figures 2 and 3.—Distribution of keratoacanthoma. Analysis of 206 lesions. The hand in text-figure 3 should be reversed. The lesions occur only on the dorsal surface of the hand, but on both aspects of the forearm.


SEX DIFFERENCES IN DISTRIBUTION
FEMALE
MALE
33 %
0 %
33 %
0 %
24 %
11 %
19 %
12 %
Other sites—no significant differences.

TEXT-FIGURE 4.—Sex differences in the distribution of keratoacanthoma.


COMPARATIVE DISTRIBUTION
[percentages]
keratoacanthoma
s.c.c.
keratoacanthoma
s.c.c.
1
0
9
4
7
0
7
18
20
0
27
4
7
34
30 lower
4 upper
5
4
16
12
keratoacanthoma 1
various covered sites
s.c.c. 24

TEXT-FIGURE 5.—Comparative distribution of keratoacanthoma and squamous carcinoma.

which the nose and cheeks are involved deserves emphasis. The lesions on the lips are equally divided between the upper and lower lip.

There are significant differences in the distribution of keratoacanthoma in males and females (text-fig. 4). While in males 11 percent of lesions occurred on the ear and 12 percent on the lips, we observed no lesions in these sites in females.

In text-figure 5 the distribution of keratoacanthoma is compared with that of squamous carcinoma. The most significant differences are the greater frequency of carcinoma on the lips, where in our cases it invariably arose on mucous membrane, and the rarity of carcinoma on the nose, a favored site for keratoacanthoma. Also significant is the greater rarity of keratoacanthoma on covered skin.

In all our cases and in all those reported in the literature the lesion was never confined to the mucous membrane, but always involved adjacent skin to a greater or lesser extent. We have never been able to exclude the possibility that the lesion arose in skin rather than in mucous membrane. In one published case (*23*) and in one of our own, the lesion arose on the alar mucous membrane of one nostril, but this is an area in which vibrissae are present.

HISTOLOGY

Since the keratoacanthoma normally undergoes rapid growth followed by progressive spontaneous involution, the histological appearances of the individual lesion must be carefully related to the stage of its evolution. This is less readily achieved than we at first believed. Although the stage of active growth rarely exceeds 6 weeks, it may be considerably shorter and though involution may begin as soon as active growth ceases, it may be delayed for months. However, though a rigid chronology cannot be imposed, the evolution of the keratoacanthoma may be conveniently studied at three stages: 1) active growth, 2) maturity, and 3) regression.

Careful examination of the sections under low magnification is often of particular value. The distinctive architecture of the lesion often suggests a correct diagnosis that excessive preoccupation with cellular detail under higher power might tend to obscure.

Stage of Active Growth

The opportunity for histological examination of the very early lesion seldom arises. One such is described (*24*) as a monstrous follicle with a central horny core surrounded by proliferating epithelium (fig. 4). By the 2d or 3d week when the distinctive pattern is well established, it is compact, circumscribed, and superficial, not extending below the level of the sweat glands. A central mass of horn is surrounded by columns of hyperplastic epithelium, whose growth is invasive, but orderly, uniform, and symmetrical (fig. 5). The basement membrane is irregular only where it is invaded by inflammatory cells. The columns are formed of large, pale cells, staining more deeply only in their outermost layers. Their nuclei are large and vesicular, with prominent nucleoli. Mitoses are numerous. They contain more glycogen, in fine granules (fig. 6), than the cells of squamous carcinoma (*25*, *26*). The surrounding surface epithelium may be normal or mildly acanthotic. Characteristically, this epithelium is raised on each side by the lateral bulging of the crater to form the so-called buttress or spur (fig. 7). Neighboring follicles may show mild epithelial hyperplasia and may contain excess horn. The dermis may appear normal, but is often edematous with an infiltrate of polymorphonuclear leukocytes, lymphocytes, histiocytes, and occasional plasma cells and eosinophils.

Stage of Maturity

The central horny mass has enlarged and keratinization extends increasingly into the columns of epithelial cells (fig. 8). The cells bordering the crater and its prolongations are large, eosinophilic, and vacuolated with pyknotic nuclei. The cellular infiltrate in the dermis is more abundant and histiocytes are relatively more frequent.

Stage of Regression

Keratinization continues (fig. 9). The crater and its extensions flatten and the horn is shed. The vacuolated cells are replaced by a granular layer. The epithelial columns are increasingly invaded by inflammatory cells, and granulomatous foci, sometimes with giant cells, are often conspicuous. These foci are first seen when fragments of keratin break through into the dermis (fig. 10). Fibrosis develops beneath the site of the lesion as the surface epithelium returns to normal.

MALIGNANT TRANSFORMATION

There are many reports of the transformation of a keratoacanthoma into squamous carcinoma, but it is extremely difficult to assess how frequently this occurs. As one's experience of these lesions increases so does one's confidence in accepted histological criteria of malignancy decline. A large proportion of the reports of malignant transformation concerns either lesions of the lips (*27*) or recurrences of lesions in any site. In almost every case the diagnosis of malignancy was necessarily made solely on histological grounds. The anatomical configuration of the lip is such that invasion of muscle fibers occurs with a relatively superficial lesion, which is still capable of spontaneous regression (*28*). Recurrences are usually subjected to biopsy at an early stage even when a typical keratoacanthoma may be difficult to diagnose histologically. It is ethically unjustifiable to leave the suspicious lesion untreated, but we have records of patients in whom there has been accidental delay in initiating treatment and in whom apparently malignant lesions have, in fact, regressed.

There is no doubt that malignant transformation of keratoacanthoma can occur in the experimental animal (*29*), but at present only clinical evidence of invasiveness can establish that it has done so. We have no record of such an occurrence in man, but the lack of the necessary proof may be a measure of the efficacy of early medical treatment.

HISTOGENESIS

Studies on Human Material

The probable derivation of the keratoacanthoma from the hair follicle was suggested by MacCormac and Scarff in 1936 (*1*), has been confirmed by many later investigators, and is suggested by distribution. Kalkoff's (*30*) careful studies of serial sections of an early lesion showed the derivation of the columns of epithelial cells from the outer root sheath of the upper part of hyperplastic follicle and from contiguous surface epithelium.

Experimental Aspects

Chemical carcinogens.—The earliest workers on experimental tar carcinogenesis noted that a proportion of the induced tumors underwent spontaneous regression. Rous and Kidd (*31*) provided a useful discussion of the status of these lesions. "Their cells have an abnormal capability for proliferation, an attribute of neoplastic cells according to Ewing; but this capability is only exerted under favouring conditions. For unless tarring is kept up or has already induced chronic changes in the supporting tissues, or unless other encouraging local conditions happen to be present . . . all of the warts eventually dwindle and disappear except those few which become cancerous."

The close similarity, clinical and histological, between the experimental papilloma and the human keratoacanthoma occurred independently to several authorities (*3, 32–34*) and stimulated further investigation. Ghadially (*34*), using repeated applications of a 1 percent solution of dimethylbenzanthracene, induced multiple tumors in 11 of 12 rabbits. Application of the carcinogen was stopped as soon as the tumors appeared. The tumors were of two main types, papillomas derived from surface epithelium and keratoacanthomas, derived from hair follicles and indistinguishable in their course and histological features from the spontaneous human keratoacanthoma. Later the same worker (*29*) employed the same experimental technique in hamsters and mice with similar results, but he found that while most keratoacanthomas in the rabbit regress spontaneously, a higher proportion in the hamster and the mouse grow continuously until the death of the animal. Lesions resembling keratoacanthomas have also been produced experimentally in chickens (*35*).

The follicular origin of the experimental tumors has been conclusively established (*36*, *37*). It was noted by Yamagiwa and Ichikawa (*38*) who, in their classic paper in 1918, used the term *folliculoepithelioma.* The concept that the phase of the hair cycle might influence the response to carcinogens is not new (*39*), but its elaboration had to await advances in our detailed knowledge of the cycle itself. Borum (*40*) found that, in 100 mice after a single application of dimethylbenzanthracene, tumors developed in none of the 55 in which the follicles were in anagen at the time of application, whereas they developed in 88.7 percent of the 45 in which the follicles were in telogen. Liang and Cowdry (*41*) also have related differences in response to the phase of the cycle. Fluorescence studies (*42*) have demonstrated that the follicles are the portal of entry for methylcholanthrene and for other lipide-soluble carcinogens. Hair follicles in telogen hold the carcinogen 10 times longer than follicles in anagen, and it remains in closest contact with the epithelium of the upper part of the follicle (*43*). Ghadially's (*37*) investigations have shown that the majority of keratoacanthomas in the experimental animal are derived from this upper part of the follicle, but that a distinct type of keratoacanthoma arises from the lower part of the follicle; we have not yet convinced ourselves that this type occurs in man. Ghadially (*37*) finds the deeper keratoacanthoma shows the most rapid and consistent regression and relates this behavior to the rapid growth and regression shown by this part of the follicle during the normal cycle. The keratoacanthoma derived from the upper part of the follicle also regresses, but less rapidly and more erratically. Although the epithelium of the upper part of the follicle undergoes cyclic changes, it does not participate directly in the hair cycle. Ghadially thus regards the keratoacanthomas as perverted products of the hair follicles, usually still under the control of the same forces that govern the growth and regression of epithelium from which they are derived.

Virus Studies

The presence of a virus has not yet been conclusively demonstrated in keratoacanthoma. Intranuclear virus-like particles have been detected with the electron microscope (*44*), and cell-free extracts from biopsy material have induced inflammatory changes and parakeratosis of ectodermal epithelium with eosinophilic inclusions in chick chorio-allantoic membrane (*45*). Other investigators (*46*) have failed to find inclusions and have obtained no positive cultures on HeLa cells.

DISCUSSION AND CONCLUSIONS

The keratoacanthoma is a common tumor derived usually from the outer root sheath of the upper third of the hair follicle, and contiguous surface epithelium, and possibly occasionally from the lower part of the follicle. Both in man and in the experimental animal the tumor may be induced by certain known carcinogenic agents. The keratoacanthoma manifests only a limited capacity for growth and a strong tendency toward spontaneous regression, possibly because the structures from which it is derived remain under the control of those influences which maintain the normal hair cycle. Both in man and the experimental animal the keratoacanthoma may show the histological features of malignancy, and be indistinguishable from a squamous carcinoma, but may still be capable of regression. In the experimental animal, with continued application of the carcinogen, an increasing proportion of the tumors fail to regress and show clinical evidence of malignancy. An analagous situation prevails in the case of the pitch worker, or, more insidiously, with slowly progressive degenerative changes. As most pitch workers are, themselves, aware, most "pitch warts" heal spontaneously, but after years of exposure some will fail to do so. It is possible that with increasing age an increasing proportion of human keratoacanthomas fail to regress, but of this we have as yet no proof. Most squamous carcinomas are not initially keratoacanthomas, but arise in areas of surface epithelium not under follicular influences and already presenting evidence of irreversible damage clinically manifest as keratosis. Many of the same factors are concerned in the induction of keratoacanthoma and of squamous carcinoma. It is surely possible that more prolonged exposure to these factors is required to induce changes in surface epithelium than in follicles. It has been demonstrated experimentally that relatively little chemical carcinogen is taken up by cells of surface epithelium (*42*) and that nonhairy skin is refractory to carcinogens (*47*).

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PLATES

PLATE 22

FIGURE 1.—Typical mature keratoacanthoma—duration approximately 8 weeks.

FIGURE 2.—Large keratoacanthoma of back of hand.

FIGURE 3.—Characteristic scar remaining after spontaneous resolution.

PLATE 23

FIGURE 4.—Very early lesion showing group of hyperplastic follicles distended with horn. Hematoxylin and eosin. × 6.5

FIGURE 5.—Mature lesion. The central horny mass is surrounded by columns of orderly and symmetrical epithelial hyperplasia. Hematoxylin and eosin. × 6.5

FIGURE 6.—Glycogen granules in hyperplastic epithelium at base of keratoacanthoma. PAS. × 100

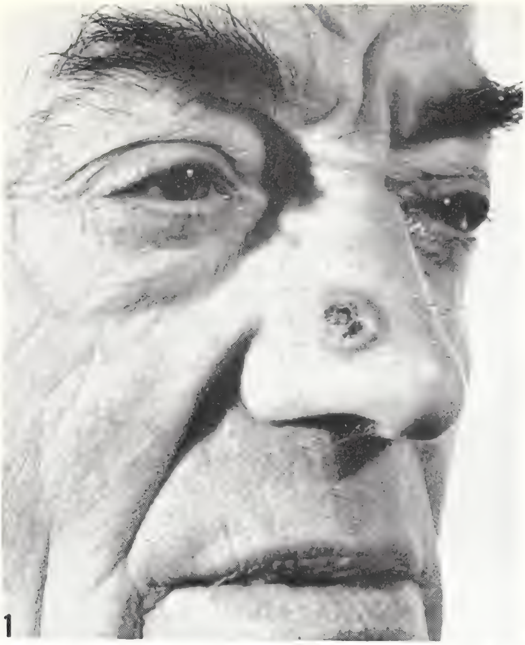
FIGURE 7.—Bulging of crater gives rise to buttress appearance. Hematoxylin and eosin. × 20

PLATE 24

FIGURE 8.—Central horny mass has enlarged and keratinization extends into columns of epithelial cells. Hematoxylin and eosin. × 6.5

FIGURE 9.—Keratinization has continued and extensions of crater are progressively obliterated. Inflammatory reaction is more intense. Hematoxylin and eosin. × 6.5

FIGURE 10.—Granulomatous focus with giant cells around fragment of keratin. Hematoxylin and eosin. × 100



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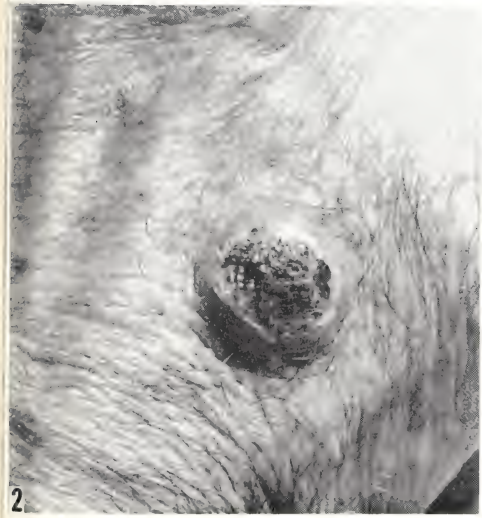

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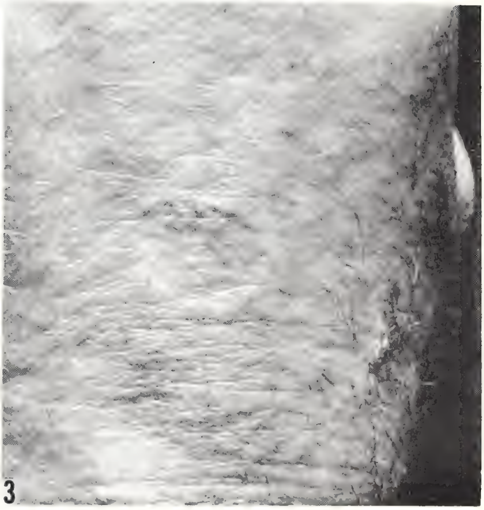

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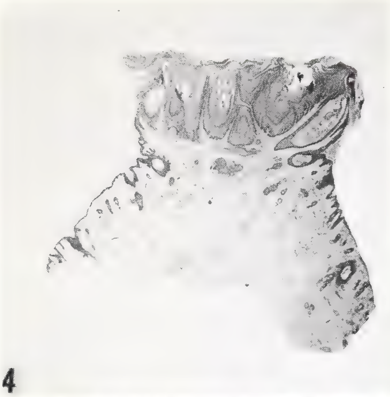

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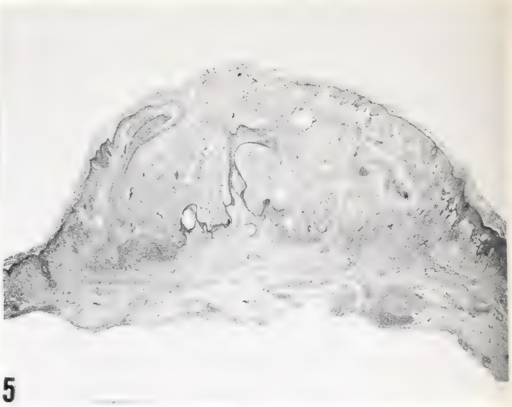

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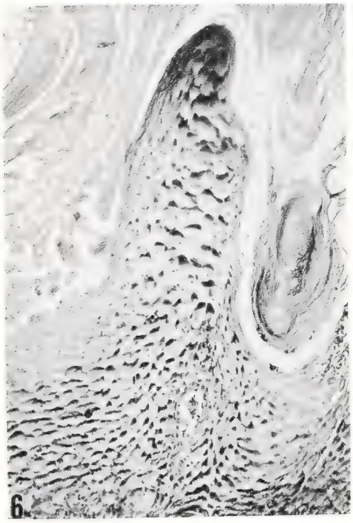

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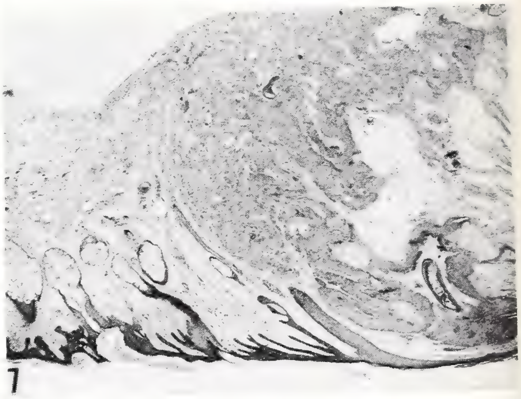

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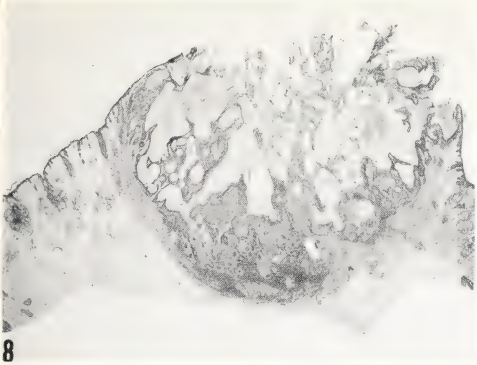
ROOK AND CHAMPION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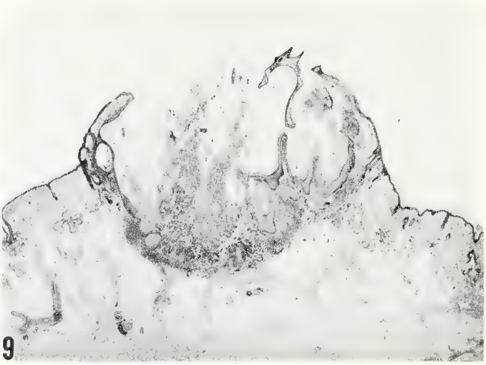
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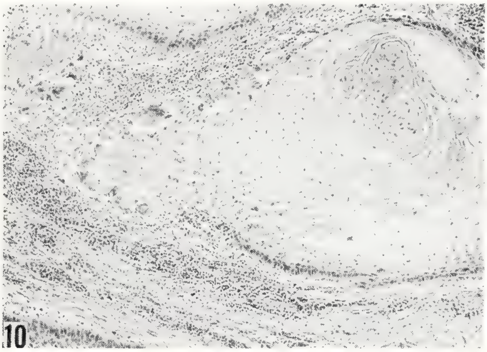
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MYCOSIS FUNGOIDES[1]

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SUMMARY

From 1953 through 1960, 38 patients with the clinical and histopathologic diagnosis of mycosis fungoides were observed at the National Cancer Institute. During this period 21 patients died; necropsies were performed on 17 of these. In the majority of patients the disease began most often in the third to sixth decades of life, as nonspecific or psoriasiform dermatoses which preceded the diagnosis of mycosis fungoides by periods of 6 months to 48 years. In 22 patients mycosis fungoides was first diagnosed in the pretumor stage. After the onset of cutaneous disease, most (22 of 38) patients developed lymphadenopathy, usually generalized. Biopsy or postmortem material from enlarged lymph nodes of 22 patients was examined microscopically. In 10 instances the diagnosis of dermatopathic lymphadenopathy was made. In 6 instances lymphomatous neoplasms were diagnosed: lymphosarcoma (4 instances), Hodgkin's granuloma, and reticulum-cell sarcoma. In 6 instances changes were present in the nodes which were sampled sequentially, initial diagnosis of dermatopathic lymphadenopathy was ultimately followed by diagnoses of lymphomas. Of the 17 patients who died and were autopsied, 14 were found to have involvement of the viscera by lymphomatous infiltrates. Cardiac and pulmonary infiltrates were sometimes detected clinically.—NCI Monogr. No. 10: 275–280, 1963.

MYCOSIS FUNGOIDES is an uncommon malignant disease of the reticuloendothelial system. It originates in the skin and may involve the lymph nodes and internal organs. Three clinical types have been described:

1) Between 1806 and 1832 Alibert of the Hospital St. Louis, Paris, described cutaneous tumors which developed in patients with various chronic lichenoid eruptions; he originated the term "mycosis fungoides" and considered the disease malignant (*1–3*). Bazin clearly characterized the eczematid, infiltrative, and tumorous stages of that type of mycosis

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fungoides which has come to be known as the type of Alibert and Bazin (*4*). Subsequently, Gillot (*5*) and Ranvier (*6*) described their microscopic recognitions of reticular lymphoid tissue in the cutaneous lesions of mycosis fungoides.

2) In 1885 Vidal and Brocq described a second clinical type, mycosis fungoides d'emblee. In patients with this type of disease the tumors are the first cutaneous manifestation; the tumors are not preceded by eruptions or infiltrates (*7*).

3) In 1892 Hallopeau and Besnier added the third clinical type of mycosis fungoides, the erythrodermal type. In patients with this type of disease there is an initial generalized erythroderma which is ultimately followed by tumors (*8*).

The description of three clinical types of malignant lymphomatoid disease as mycosis fungoides has been repeatedly criticized. Symmers suggested that the term mycosis fungoides be discarded and that diagnoses of appropriate lymphomas be substituted (*9*). Bluefarb suggested that only the Alibert-Bazin type be considered mycosis fungoides. He believes that the d'emblee type is metastatic lymphoma of the skin and that the erythrodermal form is identical with the dermatitic or dermatomyositic reactions sometimes seen in epithelial and lymphomatous cancers (*10*). Extensive reviews of their recorded experience with mycosis fungoides have been written by Herheimer and Martin (*11*), Nanta (*12*), Bluefarb (*10*), and Paltauf (*13*).

No satisfactory statistics concerning the incidence or age and sex distribution of mycosis fungoides in geographic areas or in defined populations are known to us. All statistics gathered to date are based on collections of cases from published reports. From these (*10, 14*) it seems that men are more frequently affected than women, particularly with the "d'emblee" and "erythrodermal" types. Although mycosis fungoides of all types has been described infrequently as occurring in the second and third decades, the disease is mostly seen in patients between 40 and 70 years of age.

Cameron has reported one instance of familial mycosis fungoides in a mother and daughter (*15*).

The etiology of mycosis fungoides is unknown. Experimental observations have been relatively few and inconclusive. Zurhelle (*16*) and Stratton (*17*) have produced disease in guinea pigs or mice by inoculating tissues or cultures of tissues from lesions diagnosed as mycosis fungoides. In none of this work has Koch's postulates been satisfied nor has a specific causative agent been isolated and maintained.

In the years 1953 through 1960, 38 patients with the clinical and histopathologic diagnosis of cutaneous mycosis fungoides were hospitalized at the National Cancer Institute; 21 patients died and 17 were autopsied. The histopathologic diagnosis was based on the finding of characteristic intraepidermal Pautrier's abscesses and a pleomorphic intradermal infiltrate containing atypical reticular cells. Disseminated mycosis fungoides was diagnosed when pleomorphic cellular infiltrates, similar to those seen in the skin, were found in lymph nodes prior to death or in internal organs at autopsy.

Thirty-three patients with variously diagnosed cutaneous eruptions developed lesions which could be diagnosed as mycosis fungoides; that is, their disease was of the Alibert-Bazin type. Nonspecific dermatitis and psoriasis were the most common of these premycotic eruptions; some of the eruptions were at various times diagnosed urticaria, eczema, and neurodermatitis. The range of duration of the eruptions was ½ to 48 years; the mean was 7½ years. In the majority of patients the eruptions began within the third to sixth decade. In 22 patients a diagnosis of mycosis fungoides was made during the infiltrative stage of the disease before cutaneous tumors had developed. In 16 patients, including 5 with the d'emblee type of Vidal and Brocq, tumors were present at the time of diagnosis. Twenty-two patients were men. Seven patients were Negroes.

The most frequent clinical sign other than cutaneous eruptions and tumors was lymphadenopathy, which was usually generalized. The enlarged nodes were nontender, firm, and freely movable. Excisional biopsy or postmortem examination of lymph nodes from 22 patients was diagnosed histopathologically as follows: 6 had mycosis fungoides of the lymph nodes; 4 lymphocytic lymphoma; 1 Hodgkin's granuloma; 1 reticulum-cell sarcoma; and 10 had dermatopathic lymphadenopathy, reticulum-cell hyperplasia, and chronic lymphadenitis.

In 6 patients sequential lymph-node examinations were made. The initial diagnosis in every instance was dermatopathic lymphadenopathy. But in every instance, within a 2-year period, the result of a second examination was a diagnosis of one or another malignant lymphoma. In these 6 patients, mycosis fungoides of the skin antedated the appearance of lymphadenopathy and persisted and progressed before, during, and after the development of malignant lymphomatous lymphadenopathy.

Hematologic changes in patients with mycosis fungoides are the results of not only the disease but also of its treatment and complications, which include cutaneous and systemic infections, radiation therapy, chemotherapy, and poor nutrition. Six patients were anemic, 19 had eosinophilia, and 8 had leukocytosis. In 5 the leukocytosis was associated with severe cutaneous infections; in 3 the white blood counts varied, without reason, between 10,000 and 50,000 cells per mm^3. There were large numbers of abnormal lymphoid cells, similar to those described by Sezary, in the peripheral blood smear. In 1 of these patients similar cells were seen in the aspirated bone marrow; at autopsy, no internal lesions of lymphoma were found and no abnormal cells were seen in sections from the bone marrow. In the other 2 patients, no abnormal cells were seen in bone marrow aspirates, and these cells disappeared from the blood during and following chemotherapy with nitrogen mustards which was associated with clinical improvement of the cutaneous disease.

Routine electrophoretic studies of the serum of the 38 patients did not reveal abnormalities of the plasma proteins. In 6 of the 17 patients who were autopsied, the hearts contained infiltrates of mycosis fungoides. In only 1 patient was a cardiac metastasis suspected clinically. She developed atrioventricular block which persisted for 3 months. She expired with the electrocardiographic abnormalities of nodal rhythm, auricular flutter, and atrioventricular block. At autopsy a nodular lymphosarcomatous tumor was found at the junction of the interatrial and interventricular septa.

Mycosis fungoides of the lungs was more frequently detected clinically than involvement of any other internal organ. The association of cutaneous mycosis fungoides with pleural effusions, mediastinal lymphadenopathy, infiltrates or masses in the lungs frequently led to correct clinical diagnoses.

Twenty-one of the 38 patients died during the period of this study. Of the 17 who were autopsied, 14 died with visceral infiltrations; in 3, mycosis fungoides was confined to the skin.

There are some useful prognostic signs in the care of patients with mycosis fungoides. Until tumors appear in the skin, prognosis is relatively good and some patients live many years after the appearance of infiltrates and Pautrier's abscesses, with or without X irradiation or chemotherapy with nitrogen mustards. The prognosis after dissemination to lymph nodes or internal organs is poor; in our experience patients survive less than 2 years. A frequent serious complication is infection. Seven of the patients studied died with septicemia. Six others died with severe bronchopneumonia. Hemolytic *Staphylococcus aureus* was the organism most frequently cultured from the lungs and blood. Candidal and pseudomonal septicemias also occurred. One patient expired with disseminated cytomegalic inclusion disease.

In 2 patients mycosis fungoides was associated with other cancers; both gave a history of exposure to arsenic. One patient had arsenical keratoses and cancers of the skin of the hands. He owned a small citrus orchard which he sprayed with arsenical insecticides. The other patient was given organic arsenicals as a young adult. Twenty years later she developed squamous cancer of the cervix which was successfully treated by hysterectomy. When she was 50, it was discovered she had mycosis fungoides. During the 3 years she was under treatment at the National Cancer Institute she had dissemination of mycosis fungoides to the lungs, heart, kidneys, and lymph nodes, squamous carcinoma of the anus, and carcinoma *in situ* of the vagina.

Reports of the association of mycosis fungoides with other cancers are relatively few. Bluefarb has collected *(10)* reported cases and added 2 from his practice. The other cancers include: squamous carcinoma of the skin *(3)*; carcinoma of the breast; adenocarcinoma of the rectum *(2)*; carcinoma of the cervix; carcinoma of the cervix and adenocarcinoma of the rectum; adenocarcinoma of the sigmoid colon; carcinoma of the scrotum; basal-cell carcinomas of the skin *(2)*; Kaposi's carcinoma *(3)*; lymphocytic leukemia *(2)*, and myelocytic leukemia.

Opinions as to whether mycosis fungoides is a distinct nosologic entity are numerous and varied. It is clear that there is a group of patients who develop pleomorphic lymphomas of the skin after chronic cutaneous eruptions. From the available clinical and pathologic data, the lymphoma appears to be primary in the skin. Once lymphadenopathy develops, the prognoses of the patients are similar to those of patients with other lymphomas. The disease of these patients is diagnosed as mycosis fungoides, and the name probably will be retained by dermatologists and some pathologists until they are presented with more reason to change it than has been given. It is most difficult to demonstrate the primary origin of "d'emblee" and "erythrodermal" forms of mycosis fungoides; whether these tumors begin internally or in the skin is debatable.

As more evidence for the infectious theories of the etiology of tumors is gathered and published, it is likely that there will be more clinical investigation of mycosis fungoides, for in this disease an accessible lymphoma, capable of measurement and repeated biopsy, develops slowly. But the pathogenesis of mycosis fungoides may be very complicated and depend on unknown immunologic reactions to physical and chemical carcinogens, because exposures to X irradiation, arsenic, bismuth, and tar ointments are frequently recorded in the histories of patients suffering from this affliction (*10*).

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KAPOSI'S SARCOMA IN AFRICA[1, 2]

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SUMMARY

Kaposi's sarcoma is undoubtedly common among Negroid races of Africa, and the incidence varies from 1.6 to 3.1 per 100,000 (standardized to the U.S. population of 1950). Ratio studies show a profound variation from between regions, and in the Northeastern Congo it reaches 12.8 percent of all cancers examined histologically. No cases associated with leukemia or lymphoma have been recorded in Negroids; in other respects, the disease resembles that seen in Western races. It is concluded that unknown environmental factors must be responsible for the frequency of this disease in Africa.—NCI Monogr. No. 10: 281–285, 1963.

OVER THE last 30 years a number of workers have detected an extraordinary frequency of Kaposi's sarcoma in African territories. The first cases were reported from Lagos, Nigeria, by Smith in 1932 (*1*) and in the following year by Smith and Elmes (*2*). Since then, however, others have published extensive series of cases of Kaposi's sarcoma from Africa, so that, *e.g.*, the combined experience of Murray and Lothe (*3*) and Thijs (*4*) represents about a third of the world total of cases.

It is evident that Africa offers a great opportunity for investigating this disease, its etiology, course, and therapy. The material presented here summarizes the relevant sections of the proceedings of the Kampala Conference on Kaposi's sarcoma of the Unio International Contra Cancrum in May, 1961, and a publication presented there (*5*).

The disease in Africa appears to be the same as elsewhere, both in histopathological appearance and anatomical distribution, but the absence to date of Negro and Bantu cases coexistent with lymphomas or leukemia is still inexplicable. The nature of the disease remains mysterious, although most workers consider it deserves to be classified as a multicentric malignant neoplasm. There is no obvious primary growth

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

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with later dissemination but instead a multicentric, usually symmetrical, distribution, starting with peripheral involement of skin and deeper tissues including bone and followed later by proximal and central development. Local spontaneous regression is frequently noted and occurs more commonly than with other malignant neoplasms. The curve of age-incidence of Kaposi's sarcoma is similar to that of the lymphomas with which this condition has many points in common in regard to multicentricity and organ distribution (skin, lymph nodes, bones, small intestine).

A high frequency of Kaposi's sarcoma in certain areas apparently is not a recent development. A survey of the histopathological material at the South African Institute for Medical Research from 1911 to 1927 (*5*) revealed that Kaposi's sarcoma was observed in 1912. Further secular studies of case accessions since 1947 do not suggest any recent increase in Johannesburg nor is this evident in Uganda.

RACIAL DIFFERENCES

The racial susceptibility to Kaposi's sarcoma has always remained one of the striking clinical features of the disease in Western communities. In Africa it is far commoner among the Negroes than in other populations living alongside them, *e.g.*, in South Africa it is rare among the Indians of Natal (*6*), the colored (mixed) of Cape Town (*7*), or the whites (*5*). These populations, although in close contact with the Negro groups, remain relatively unaffected. It seems somewhat more common among rural than among urban Bantu in the Transvaal (*8*), but since it is easier to biopsy cases of Kaposi's sarcoma than those in the deeper tissue, possibly this difference may be explained as an artifact of sampling for histological examination.

The incidence of Kaposi's sarcoma has been estimated in 3 centers to date, viz., Johannesburg, South Africa (*5*), Lourenço Marques, Moçambique (*9*), and Kampala, Uganda (*10*). Curves of age-incidence show that this condition starts at an early age, and rises steadily thereafter. As has been noted, the slope of the curve closely resembles that for lymphoma. The rates obtained are based on small numbers and are best compared as standardized to the U.S. population of 1950 (*11*) or the proposed African Standard Population (*12*). These are given in table 1. It is noted that the figure in Johannesburg is about half that obtained in Uganda, while that of Moçambique approaches that of Uganda. Comparison with the figures for U.S. whites and Negroes shows that the African incidence in Johannesburg is increased 20 times and in Uganda 40 times by comparison with the U.S. white rates. U.S. Negro incidence, however, approximates that of U.S. whites.

Comparisons between the different African countries have for the most part been based on a relative frequency of diagnosis in various histopathological series (table 2). In most of these series the total

TABLE 1.—Kaposi's sarcoma: crude morbidity rates per 100,000 per annum in various regions

Region	Crude rates		Male rate standardized to African standard population	Male rate standardized to U.S. standard population, 1950
	Male	Female		
West Africa, Negro	0.37	—	—	—
South Africa, Johannesburg Bantu	1.00	—	1.1	1.6
Uganda, African	2.6	—	2.6	3.1
Moçambique, Bantu	2.2	—	2.4	2.9
U.S., white	0.081	0.016	—	—
U.S., Negro	0.103	—	—	—
Sweden	0.28	0.04	—	—

TABLE 2.—Kaposi's sarcoma and lymphomas in Africa: percentages of all cancers in histopathological series in Negroes (both sexes) in African territories

Region	All cancers	Kaposi's sarcoma	Percent	Lymphomas	Percent
Tunisia	2, 818	1	0. 04	—	—
French West Africa	170	2	1. 2	229	12. 2
	1, 884	5	0. 3		
Gold Coast	1, 193	10	0. 8	104	8. 7
Nigeria	500	10	2. 0		
	1, 000	24	2. 4	53	5. 3
Uganda	204	10	4. 9		
	734	25	3. 4	56	7. 6
Kenya	50	2	4. 0		
	3, 000	73	2. 4		
Tanganyika	662	22	3. 3	89	13. 4
French Equatorial Africa	—	18	5. 0	—	—
Belgian Congo	94	12	12. 8		
	2, 529	230	9. 1	330	13. 0
Rhodesia	334	2	0. 6	—	—
Transvaal, Johannesburg residents	497*	7	1. 4	26	5. 2
	693	8	1. 2	41	5. 9
Johannesburg nonresidents	564	6	1. 1	34	6. 0
Rural	212	6	2. 8	13	6. 1
Natal	1, 921	25	1. 3	97	5. 0
Moçambique	1, 273	23	1. 8	97	7. 6

*Three cases of lipomelanotic reticulosis excluded. [References given in (*5*).]

incidence of cancer is much lower than that for westernized communities, *e.g.*, in the Johannesburg Bantu the cancer incidence is only half that of an equivalent population of U.S. whites or Negroes (*8*), and in Uganda one third of the expected figure (*10*). This naturally inflates the histological ratios of Kaposi's sarcoma to all cancers twofold or threefold.

African populations also contain a larger proportion of the younger age groups, so that cancers commencing at an early age seem to be more frequent. Accessibility to biopsy increases the ratios still further, as does the chronic nature of Kaposi's sarcoma. Using the African ratios available (*5*) (table 2), one can see that the peak relative frequency is in the Northeastern Congo where it may reach 12.8 percent. It falls off to 4.5 percent in Uganda, Kenya, and French Equatorial Africa; to 1.2 percent in the Transvaal, Lourenço Marques, and Nigeria; and to under 1 percent in French West Africa, the Gold Coast, and Tunisia. In these latter regions it would seem that the frequency of Kaposi's sarcoma approaches that of the United States.

SEX RATIO

The greatly increased tendency of Kaposi's sarcoma to affect males is seen also in African patterns. Remarkably enough, however, the masculinity varies from 1,833 [Uganda, (*13*)], 1,470 [Transvaal, (*5*)], 860 [Congo, (*4*)] down to 213 for the Durban Bantu (*6*). Even more striking is the fact that in South African whites, female patients exceed male (masculinity, 80) (*3*), and the same is noted in Algeria (masculinity, 67) (*14*). African studies show that the masculinity is of the same order before puberty, during the reproductive life, and after the menopause, which suggests that the levels of circulating sex hormones probably are not the main factor here. Sex ratios of this order are not necessarily endocrine or genetic, especially in view of the low levels recorded for some groups, and environmental reasons seem the most likely explanation.

In children, Kaposi's sarcoma confined to the lymph nodes has been reported several times from Africa (*15–19*) and (*13*). Histologically, the disease seems exactly the same as that occurring in other sites and involvement of the skin may occur early, late, or not at all in individual children.

EVIDENCE OF ETIOLOGICAL FACTORS

The striking differences in susceptibility to Kaposi's sarcoma seen in the various races of Africa, and within any one race in the various regions of Africa, suggest there may be many precipitating and predisposing factors. While an inherited competence is essential in the development of any disease, it would seem unlikely that the increased susceptibility of African Negroid races can be regarded as primarily genetic in view of the relative insusceptibility of American Negroes. The site distribution of the tumors and the sex distribution of the disease suggest the predominant factors are environmental, as does the extraordinary variation between different regions of Africa, *e.g.*, even within the Belgian Congo. To date, however, no satisfactory or even convincing hypothesis has been put forward and a further intensive investigation of etiological factors by techniques of retrospective questioning and virology are indicated.

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MALIGNANT MELANOMA OF THE SKIN IN MAN[1]

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SUMMARY

In a study of 392 patients with malignant melanoma, 96 percent were Caucasian. There were only 4 Negroes and the melanoma in each of these involved either the sole of the foot or palm of the hand. Among the Caucasians, 30 percent had melanoma on the trunk, 25 percent on the lower extremities, 23 percent on the head and neck, and 22 percent on the upper extremities. Eighty percent of the patients claimed origin of the melanoma in a preexisting nevus. In small lesions, the diagnosis of malignant melanoma must be made on the basis of several microscopic changes in a technically perfect histologic section. Malignant melanoma arising in a melanotic freckle of Hutchinson may have a better prognosis. Spindle-cell and epithelioid-cell nevus occurs not only in children but also in adults. This type of nevus biologically follows a benign course. The term "juvenile melanoma" should not be used for this lesion because it might cause unjustified surgical mutilation. The cellular type of blue nevus is sometimes mistaken for malignant melanoma. Four of 192 cellular blue nevi metastasized only to regional lymph nodes, and the patients are alive and apparently well many years after excision of the involved lymph nodes.—NCI Monogr. No. 10: 287-295, 1963.

IN RECENT years the natural history of pigmented lesions of the skin has been more clearly defined. This is emphasized by the fact that certain lesions formerly considered malignant are now regarded as benign. In this preliminary study some clinical and pathologic features of malignant melanoma, spindle-cell and epithelioid-cell nevi, and cellular blue nevi will be discussed.

MALIGNANT MELANOMA

The present discussion is based on data obtained from the first 392 of 3,000 cases of malignant melanoma of the skin recorded at the Armed Forces Institute of Pathology (AFIP). Initially, the specimens were usually surgical. Autopsy material was contributed later in many instances and lengthy questionnaires were mailed to survivors. In each case a complete microscopic examination was made.

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6-11, 1962.

Clinical Data

Race and sex.—Ninety-six percent of the patients were Caucasian. Thirteen patients of other races included 4 Negroes, 2 Mexicans, 2 Filipinos, 2 Puerto Ricans, and 3 Polynesians. The ratio of males to females was 4.8 to 1 (323 males and 67 females in the 390 patients with data). This high ratio of males to females is probably due to the predominantly male population of the various sources of the AFIP material. A ratio of 0.8 males to 1 female was reported in a group of 650 males and 797 females with histologically confirmed malignant melanoma of the skin (*1*).

Only 4 Negroes among the 392 patients are in distinct contrast with the observations of Muelling (*2*) who recorded 28 percent Negroes among 92 patients with melanoma at Charity Hospital in New Orleans. The admissions to Charity Hospital were nearly equal for white and Negro, but there was probably a degree of selectivity in each group. About 10 percent of the military population, the largest contributor of AFIP cases, was Negro. Of 862 patients with malignant melanomas, Pack, Perzik, and Scharnagel (*3*) reported only 10 Negroes.

Age.—Sixty-one percent of the patients were under 40 years of age at the first biopsy or surgical excision. This reflects the fact that the AFIP material comes largely from personnel in the Armed Forces. The youngest patient in this study was 10 years of age, the oldest 89. The median age of 34 years for this group is higher than that for the Armed Forces, an indication that patients with malignant melanoma in the general population would have an older age distribution. This is supported by data from a recent monograph based on 125,443 cases reported in 10 large States and hospital tumor registries, in which only 29 of 1,447 patients with histologically confirmed malignant melanoma were under 45 years of age (*1*).

Eye, skin, and hair color.—Data were available on 97 patients. Blue, gray, or green eyes were predominant (72%) and blond or red hair was recorded for the majority (60%). Almost four fifths (80%) of the patients had fair or ruddy complexions. Other investigators have noted similar observations (*4*).

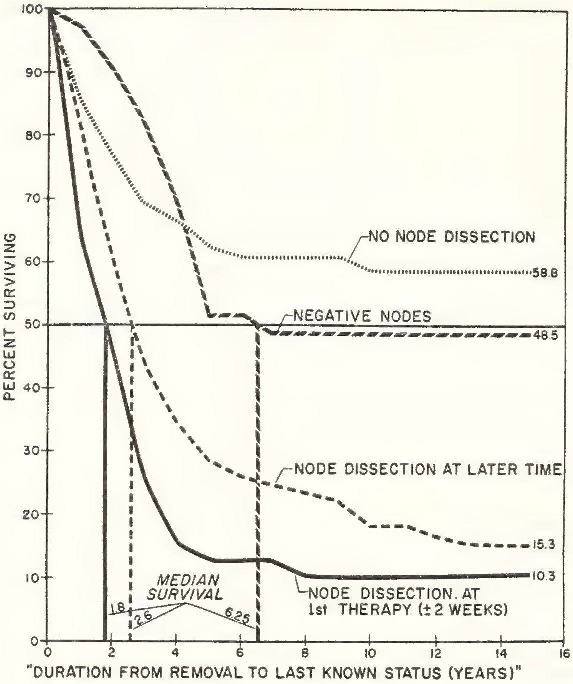
Anatomical site.—Thirty percent (118 of 392 patients) had primary malignant melanoma on the trunk, 25 percent on the lower extremities, 23 percent on the head and neck, and 22 percent on the upper extremities. The single, most frequently affected area was the skin of the back (76 cases). Eighty lesions were on the lower leg, ankle, and foot; 43 on the upper arm, forearm, and hand. This distribution is in sharp contrast to that noted by Sirsat (*5*) in Indians in whom 75 percent of the lesions occurred on the lower extremities and the greater percentage over the heel and sole. The lesions in the 4 Negroes in this study were located on the plantar surface of the foot in 2, on the skin of the toe in 1, and on the web of the thumb in the other. Morris and Horn (*6*), both in their own material and in collected material, observed that the foot and leg are the most common sites in the Negro. Van Scott, Reinertson, and McCall (*7*) noted no difference between whites and Negroes in the number of plantar nevi, but both male and female Negroes had a greater number of nevi on the palms than had the whites. Both trauma and decreased pigment on the soles of the foot have been claimed as causal factors (*8*).

Origin.—Eighty percent of all patients claimed origin of the melanoma in a pre-existing nevus or pigmented lesion. Ninety-two patients stated the melanoma arose in a nevus that had been present since birth; 41 stated it arose in a nevus of recent growth; 53 stated the nevus was of many years' duration; and 127 did not know the duration. Twenty patients associated the origin with a scar or burned area, infection, or "blister."

Statistical data did not prove any significant association between major groups of anatomic sites and origin of malignant melanoma in nevi or other types of lesions.

Geographic distribution.—The geographic distribution in the United States was tabulated for 287 Caucasian males. This distribution was based on replies of patients to a follow-up inquiry as to the State in which they had resided longest. Sixty-four percent (183 patients) had lived in States below or bordering on the 44th parallel. When States were grouped in accordance with the death rate for cancer of the skin (*1*), those in the first, second, and lowest fifth of the distribution were the same in this series as in ours. Their third and fourth quintiles were reversed in our series; however, there were only 4 more patients in the fourth than in the fifth quintiles. These data suggest that there is a relation between sunlight and malignant melanoma. Workers in Australia have made a similar suggestion (*9*).

Survival rates.—The actuarial survival rates reveal the proportion of patients who can expect to enter a given postoperative year alive. Twenty-six percent of the patients whose positive lymph nodes are removed at time of first therapy can expect to enter the 4th year alive; the same proportion of patients with nodes removed at a later time can be expected to enter the 7th year alive; the groups with no node dissection and those with negative nodes require more than 15 years to reach 74 percent mortality. The exact duration for these latter 2 groups cannot be determined as they lie outside the 15-year range of the survival table (text-fig. 1). Patients who had lymph nodes dissected at the time of the first treatment, compared with those whose lymph nodes were removed 2 weeks later, did not differ significantly in their survival time. Lane, Lattes, and Malm (*10*) noted that their patients who had only an adequate local excision without lymph-node dissection had an apparent 5-year cure similar to the individuals who had a prophylactic lymph-node dissection. This does not really indicate that local excision alone is as effective as local



Text-figure 1.—Malignant melanoma, skin. Survival rates by node dissection.

excision plus lymph-node dissection. The size and extension of the primary lesion must be considered in evaluation of the data.

Pathology

Although patients frequently claim origin of a malignant melanoma from a previously existing nevus, the microscopic evidence of a nevus in the routine histologic sections of malignant melanoma is not especially common (20% in this series). Serial sections might disclose nevus cells in a greater number of lesions, and it is also possible that the malignant melanoma might destroy pre-existing nevus cells.

Gross pathologic changes of an infiltrated, elevated, or even bulky mass that is brown or black, and in the larger tumors, ulcerated, are useful in clinically suspecting a diagnosis of malignant melanoma. The presence of satellite pigmented nodules makes a gross diagnosis of malignant melanoma even more likely. But the final interpretation must always be based on the histologic changes.

The microscopic changes in a large lesion of malignant melanoma practically always readily confirm the clinical diagnosis. A large lesion really does not present any problem in diagnosis, but in recent years clinicians have become suspicious of melanoma in smaller lesions, so that the difficulty for the pathologist in establishing the diagnosis has risen proportionately. Furthermore, the final diagnosis is always a microscopic diagnosis.

To understand the microscopic diagnosis of malignant melanoma, it is necessary to review a classification of nevi. This classification depends chiefly on the location of the nevus cells in the skin. If nevus cells are located only at the junction of the epidermis and dermis (epidermal-dermal junction), the nevus is called a junction nevus and the change may be referred to as junction change. If nevus cells are located only within the dermis, the nevus is called an intradermal nevus. If nevus cells are located both at the epidermal-dermal junction and within the dermis, the nevus may be called a compound nevus. In a study of 1,000 patients, Sirsat and Helwig (*11*) have confirmed the observations of others (*12*) that nevi in children (78% in ages 0–14) more often show junction change than do nevi in older adults (25% age 40 and over). This reference to "junction change" is important since the expression "junction change" is often used in descriptions of malignant melanoma. It must be emphasized that the expression "junction change" has little meaning in regard to benignancy and malignancy unless many other factors are known and taken into account.

Most primary malignant melanomas show groups of cells, often pigmented, situated at the junction of the epidermis and dermis (junction change). In contrast to the small, uniform cells of a junction or compound nevus, the cells in junction change associated with malignant melanoma often are enlarged, with large, sometimes bizarre, nuclei. These collections of cells may markedly expand the rete ridges.

What are the requirements for the interpretation of a lesion as malignant melanoma? Before any interpretation can be made there must be technically acceptable sections for study. Perhaps the most perplexing situation in the diagnosis of malignant melanoma is to ask the pathologist to make a decision on a thick, ragged, and poorly stained preparation in which single cells and groups of atypical cells appear at the epidermal-dermal junction. Furthermore, the sections must include all variations in morphology noted in the gross specimen.

Once the adequacy of the preparation is assured, then the microscopic changes may be evaluated. Microscopically, there is no single criterion by which a diagnosis of malignant melanoma may be made, but one change that always must be present is invasion of the corium by melanoma cells. The invasion of malignant melanoma cells must be distinguished from the observation of benign nevus cells extending deep into the corium. The cells of the benign nevus are uniform and coherent but may include multinucleated forms. Perhaps the most important feature in the diagnosis of malignant melanoma is the one most difficult to describe—the relationship of the cells to the stroma. No matter how irregular the over-all pattern of a benign nevus, the cells show an organizational relationship with the stroma. In contrast, the cells of a malignant melanoma often appear in the stroma without an organized pattern or a discernible, fixed, cellular-stromal relationship.

Now to return to the problem of the junction nevus with atypical cells. As previously pointed out, these atypical cells may tremendously expand the rete ridges, but the ridges still show a thin, compressed, and stretched layer of epithelial cells at the base, which are identified mostly by the smooth margin. If, however, the smooth margin is interrupted and a few atypical cells are infiltrating into the corium without an organizational pattern, then the lesion is interpreted as an incipient malignant melanoma. Such a lesion should be treated as any malignant melanoma is treated. If the atypical cells are confined to the epidermal-dermal junction, the lesion is interpreted as a junction nevus. No examples of junction nevus with atypical cells and metastasis have been found. Isolated groups of cells being carried up in the epidermis and desquamated are commonly observed in malignant melanoma, but this change may be seen in benign nevi and cannot be used as a criterion for malignancy. Mitotic figures as a rule are observed in malignant melanoma but also may be seen in nevi. Nuclear inclusions may be noted in both nevi and malignant melanoma. One problem that may arise in the presence of junction change and cells in the adjacent corium is the question of "maturation" of the nevus cells. If the cells in the deeper stroma are smaller and cellular atypia is absent, there is probably "maturation" and the lesion is a nevus rather than a malignant melanoma.

Ulceration is common in malignant melanoma but is infrequently seen in benign nevi and, if present, usually follows trauma. Because of this association with melanoma, all ulcerated lesions should be carefully and extensively examined for evidence of malignant change. In the present study, approximately 50 percent of the melanomas showed microscopic evidence of ulceration. Tompkins (*13*) pointed out the grave prognostic significance of ulceration.

One type of junction nevus stands apart. This is the melanotic freckle of Hutchinson (*14, 15*), a lesion that is usually readily recognized by clinicians because of its characteristic appearance, but is often overlooked by pathologists. The clinical course and gross appearance are a slowly enlarging, dark brown, nonelevated, irregular-shaped area. The face and neck are common sites, though the lesion may occur anywhere on the skin and even on the conjunctiva. Many lesions gradually enlarge for years and some develop foci of thickening often accompanied by a change in pigmentation. Before areas of thickening develop, the lesion microscopically shows junction change with large numbers of clear cells as well as theques of cells, but these features may vary within the lesion. Some thickened areas in a lesion may show changes of a compound or intradermal nevus, but in many instances the thickened areas show atypical cells infiltrating the corium and the process is then regarded as a malignant melanoma capable of metastasizing. Even so, some investigators believe that a malignant melanoma arising in a junction nevus of the melanotic freckle type is less aggressive and the patient has a more favorable prognosis. More comprehensive studies are necessary to prove this contention.

Occasionally, a junction-type nevus must be differentiated from extramammary Paget's disease, even though the existence of the latter lesion has been denied (*16*). Helwig and Graham (*17*) have recently studied 40 patients with anogenital extramammary Paget's disease and have pointed out the differences from malignant melanoma. Histochemically, the cells of anogenital Paget's disease contain a mucin material that is aldehyde-fuchsin positive, and the cells in the malignant melanoma are negative.

SPINDLE-CELL AND EPITHELIOID-CELL NEVI

Several years ago Spitz (*18*) described melanomas in childhood and pointed out that only 1 of 13 patients with juvenile melanoma in her series had a fatal course, despite the similarity to malignant melanoma of adults. She believed that the juvenile melanoma may be distinguished histologically from adult melanoma in about half the cases by the presence of giant cells in the juvenile group. For many years I have had an opportunity to study a large number of nevi in children and have observed similar lesions containing giant cells. However, more commonly the lesions were comprised of fascicles of spindle-shaped cells and in some instances both spindle-shaped cells and epithelioid cells (apparently the giant cells of Spitz) were in the same lesion. Only in exceptional instances were the lesions comprised entirely of epithelioid-type nevus cells. On the basis of the type and location of the nevus cell, I have classified this lesion as a compound spindle-cell or epithelioid-cell nevus (*19*). The term "juvenile melanoma" is unsatisfactory and misleading because melanoma has come to mean a malignant tumor, and therefore, an occasion for unjustified radical surgery may result. Furthermore, the observation of lesions of similar histological appearance in adults makes the term "juvenile" inappropriate.

Grossly, these lesions usually appear elevated, smooth, and pink more often than brown. Microscopically, the epidermis over the tumor is often strikingly acanthotic, with long and intercommunicating rete ridges that enclose masses of nevus cells. The nevus cells are usually spindle-shaped and arranged in short fascicles but the epithelioid-type cell may be large and multinucleated. As a rule, little or no melanin pigment is present. Groups of cells are usually located at the epidermal-dermal junction and sometimes extend into the deep corium. Mitotic figures may be present. In this study, the natural history of the spindle-cell and epithelioid-cell nevus in both children and adults is that of a benign lesion.

Malignant melanoma rarely occurs in children and when present biologically behaves as it does in malignant melanoma in the adult (*20*). In an incomplete study, among 10 patients with malignant melanoma, who ranged in age from 3 to 14, two age 7, one age 10, and another age 14 developed metastases. The 10-year-old patient died after widespread metastasis. Histologically, the malignant melanomas that occurred in children appeared similar to those in adults. It is obvious that the spindle-cell and epithelioid-cell nevi should be distinguished from malignant melanoma.

CELLULAR BLUE NEVUS

The blue nevus of Jadassohn–Tièche includes one group that is more cellular than most (*19*). This group is often referred to as cellular blue nevus. They occur almost exclusively in the region of the dorsum of the foot and ankle (50%), sacrum and buttocks (25%), and in the region of the dorsum of the head (20%). A few occur elsewhere on the body. In a preliminary study of 192 patients with cellular blue nevus a few claimed the lesions had been present since birth and most noted they had been of long duration. Growth was gradual except in rare instances. Usually the lesions ranged from 0.5 to 1.5 cm in diameter but occasionally they were larger. Most tumors were elevated and hemispherical but a few were flat. As a rule they were black or blue covered by smooth skin. While most patients complained of only a mass, several experienced pain, and a few observed inflammation, usually secondary to trauma. At the time of removal the youngest patient was 4 years of age and the oldest 70. Most were between 20 and 35 years of age.

Grossly, the cut surface is usually blue or blue-black in the outline of the tumor.

Microscopically, the picture shows variations, but the essential feature is the presence of irregular, sometimes intercommunicating, fascicles and coils of spindle-shaped cells containing much or little melanin as seen in the hematoxylin and eosin preparations. The spindle-shaped cells may appear bipolar in a Bodian preparation. The fascicles of spindle-shaped cells are separated by a fibroblastic stroma often containing masses of melanin, both extracellular and within histiocytes. Occasionally the fascicles are separated by a clear space from the intervening stroma, which gives a false impression that the nevus cells are within lymphatic or vascular spaces. In addition to the cellular areas there may be foci of spindle-shaped cells, usually pigmented, situated in a fibrous, collagenous background. This is the picture seen in the usual blue nevus. Occasionally it is difficult to decide whether a blue nevus is of the ordinary type or of the cellular variety since they seem to be variations of the same basic lesion. The cellular blue nevus may contain giant cells but the nuclei, while sometimes many, are uniform in contrast to the nuclei of giant cells in malignant melanoma. Mitotic figures as a rule are rare. The margins of the lesions are often irregular and because of this probably some lesions occasionally are not completely excised.

Because of the cellularity of this variety of blue nevus, occasionally it has been mistakenly diagnosed as malignant melanoma. To complicate the situation further, the cellular blue nevus rarely is associated with metastases. However, an evaluation of the natural history in this group of patients is enlightening. In 4 of the 192 patients, metastases were detected in the regional lymph nodes about the time of excision of the nevus in the skin. These 4 patients have been followed from 8 to 15 years since the removal of the metastatic lesions and all are alive without having experienced further evidence of spread. On the basis of these observations the cellular blue nevus rarely metastasizes and, when it does, usually reaches only the regional lymph nodes. It rarely, if ever, causes death of the patient.

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CARCINOGEN-INDUCED MELANOTIC TUMORS IN ANIMALS[1, 2, 3]

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SUMMARY

Carcinogen-induced melanotic tumors of the Syrian hamster differ morphologically and biologically from the spontaneous malignant melanomas of the same species. They are composed of dermal melanocytes, show no junctional changes, and resemble cellular blue nevi or pigmented neurofibromas of man. They grow slowly and metastasize rarely. They can be induced in the golden hamster, in which malignant melanoma occurs spontaneously, and in the white hamster in which malignant melanoma has never been reported. They are derived from cells that can be stimulated to grow and form melanin pigment by the topical application of 7,12-dimethylbenz[*a*]anthracene (DMBA) and the oral administration of urethan. In contrast, epidermal melanocytes of the Syrian hamster do not respond with increased melanin production or with cellular proliferation to these carcinogens. The fine structure of the pigment granules in evolution is described in detail. In tumors of the golden hamster it resembles that of normal dermal melanocytes. In tumors of the white hamster it is highly atypical, probably because of genetic differences in the response to carcinogens between white and golden hamster melanocytes. Neural elements, particularly Schwannian cells, are abundant in the early perifollicular tumors of both hamsters. Occasional melanocytes containing the characteristic pigment granules were demonstrated within small nerve fascicles electron microscopically. These observations support the hypothesis that the component cells of the carcinogen-induced melanotic tumors are of endoneurial and probably Schwannian cell origin. We concluded that, in the Syrian hamster, cells of the neuroectodermal mesenchyme of small dermal nerves are susceptible to the melanogenic and growth-inducing effects of DMBA and give rise to the carcinogen-induced melanotic tumors of this species. In contrast, the epidermal melanocyte of the Syrian hamster appears to be resistant to the melanogenic and carcinogenic effects of DMBA.—NCI Monogr. No. 10: 297–322, 1963.

THE SYRIAN HAMSTER represents an excellent experimental model for the study of melanocytic tumors which morphologically and biologi-

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cally resemble the corresponding tumors in man. In an initial observation in 1956, it was found in our laboratory that multiple melanotic tumors can be readily induced in the Syrian golden hamster by a single application of a relatively small dose of 7,12-dimethylbenz[*a*]anthracene (DMBA) to the skin of the back (*1*). These DMBA-induced melanotic tumors grow slowly, metastasize rarely, and seem to have no demonstrable effect on the life expectancy of the host. Histologically, they are composed of dendritic melanocytes located in the dermis. They usually resemble either cellular blue nevi or pigmented neurofibromas of man. Morphologically similar tumors (fig. 1) can be induced in the white hamster under identical experimental conditions.

In 1958, Fortner and Allen first reported spontaneous malignant melanomas in the Syrian golden hamster (*2*). Occasional tumors of this type have been observed in our hamster colony, but not in any of our DMBA-treated animals. In contrast to the DMBA-induced melanotic tumors, the malignant melanomas of the Syrian hamster are highly invasive, metastasize regularly, and kill the host. The dermoepidermal junction is always involved [fig. 2, (*2*)], whereas the DMBA-induced tumors are separated from the epidermis by uninvolved dermis. This correlation between histologic structure and biologic behavior is remarkably similar to that of the corresponding tumors in man.

In no other species have single applications of chemical carcinogens been reported to induce melanotic tumors. Only sporadic "melanomas" have been observed after repeated application of chemical carcinogens or carcinogenic tars in mice (*3, 4*), in dogs (*5*), in 1 rabbit (*6*), and in guinea pigs (*7, 8*). In most of these experiments, the descriptions or illustrations are insufficient to decide whether these tumors were true malignant melanomas or melanotic tumors composed of dermal melanocytes. However, the tumor reported by Burgoyne and associates in mice (*4*) is morphologically indistinguishable from the carcinogen-induced neoplasms observed in the golden hamster.

The dermal melanocyte system of the Syrian hamster is particularly susceptible to the topical application of DMBA. It appears to respond more readily to the action of this carcinogen, with tumor formation, than the epidermis and differs in this respect from the melanocyte system of other species. For example, it has been observed that a dose of DMBA that is subeffective in the mouse (an initiating dose) gives rise to melanotic tumors in the hamster; when the dose of DMBA is increased many epidermal tumors are induced (*1*). Thus it can be seen that different tumors have greatly different requirements for their emergence, even when occurring in response to the same carcinogen. The requirements for the induction of melanotic tumors differ from those of epidermal tumors in some respects. They occur in response to as little as 50 μg of DMBA in acetone (*9*), a dose well below that capable of inducing skin tumors in any of the other species tested (*10*). Many related hydrocarbons and a variety of other carcinogens, except urethan, do not give rise to these tumors (*9, 11–13*). In contrast to DMBA, urethan had to be administered orally or intraperitoneally and was ineffective when applied directly to the skin.

Numerous small melanotic spots appear about 10 weeks after a single application of 4 drops of 1 percent DMBA in mineral oil (approximately 800 μg of DMBA) to the skin of the back of the Syrian hamster. Histologically, they are composed of aggregates of melanocytes arranged around hair follicles (fig. 5). At a later stage some of them progress to tumor formation. A sharp line of demarcation between hyperplasia of dermal melanocytes and melanocytic tumors could not always be drawn, and we elected, somewhat arbitrarily, to regard those lesions which measured 0.2 cm in diameter, or more, and at the same time were distinctly elevated above the level of the skin, as melanotic tumors (*1*). In contrast to the spontaneous malignant melanomas, the induced melanotic tumors are separated from the dermoepidermal junction by a zone of uninvolved dermal connective tissue. They are composed of melanocytes, which, as a rule, do not have the cytologic features of malignant neoplastic cells. These tumors also show an abundant network of reticulin fibers that envelop virtually every tumor cell (fig. 3), while the malignant melanomas show an alveolar reticulin pattern in which groups of tumor cells are surrounded by argyrophilic fibers [fig. 4, (*14*)].

To investigate the effects of DMBA on a presumably poor pigment producer, we applied DMBA in single doses to the skin of the white hamster (*15*). The white hamster is not a true albino since pigmentation of the ears, perineum, and retina occurs normally (*16*). However, under normal conditions the rest of the skin remains nonpigmented over the entire lifespan of the animal.

Melanotic tumors were as readily produced in the white as in the golden hamster (*15–17*). They showed considerable variations in color, ranging from pale pink to dark blue-brown. Microscopically, it could be shown that the basic structure and cytology of the tumors produced in the white hamster (fig. 1) was almost identical with that of the golden variety, but that there were great variations in the number of pigmented cells and in the staining intensity of the pigmented granules. These granules were never so abundant as to obscure the cytologic features of the pigment-carrying melanocytes. That DMBA can induce pigmented tumors in nonpigmented skin suggested that DMBA in some way converts amelanogenetic melanocytes into melanin-producing cells.

In the skin of the DMBA-treated white hamsters, in addition to the tumors, we found perifollicular aggregates of melanocytes similar to those forming the pigmented preneoplastic spots in the golden hamster. In the white hamster some of those aggregates were composed entirely of nonpigmented cells [fig. 6, (*15*)]. On the other hand, Quevedo and co-workers (*18*) showed that repeated cutaneous applications of croton oil in acetone are followed by the appearance of numerous perifollicular networks of pigmented melanocytes within 15 weeks, but that in spite of continuing treatments for 26 weeks melanocyte networks retained their structural integrity without progressing to tumor formation. It would appear then that in the white hamster cutaneous applications of croton oil result in increased melanin synthesis which is associated with migration or multiplication of melanocytes but not with tumor formation, while DMBA induces a neoplastic proliferation of melanocytes, often without a commensurate increase in melanin synthesis (*14*).

As mentioned previously, in the golden hamster all melanotic tumors are heavily pigmented, and the pigment granules appear almost black under the light microscope. In the white hamster, however, the pigment is lighter and the cytoplasmic details can be readily detected. This variation is reflected in the electron microscopic appearance of the neoplastic melanocytes in the golden and white hamster, respectively (*14, 19*).

In the golden hamster melanin granules are round to short oval in profile, measuring up to about 800 mμ in diameter. In fully grown tumors the granules have a high electron density, so that details of fine structure are difficult to analyze (fig. 9). At an early stage of tumor formation, however, some of the melanin granules still retain a substructure of smooth-walled vesicles of varying sizes and electron density (fig. 10). They are elliptical and contain a longitudinally oriented, striated component made up of delicate fibrillar structures which show a distinct periodicity in the order of 90 A. Melanization appears to occur on this fibrillar component. Occasionally, nonpigmented tumor cells are also seen.

In the tumors of the white hamster, the melanin granules show great variations in size, shape, and internal structure, varying from round or oval to elongated to irregular in outline [figs. 11 and 12, (*14, 19*)]. The smallest granule measures about 0.1 μ in diameter and the largest one reaches about 6 μ in length and about 1.5 μ in thickness. Melanin is arranged in clusters of short rods (figs. 12 and 15), each measuring about 20 mμ in thickness and to about 500 mμ in length, surrounded by a granular and striated material of lower electron density. Striations frequently extend across the length of the granules (figs. 11 and 12) and sometimes produce a crystalloid appearance (fig. 13). The melanin rods appear to be arranged, in the beginning, along the striations but, later on, in a haphazard fashion without consistent relation to the striations (fig. 12). Numerous granules devoid of melanin rods also occur (figs. 11 and 16). These vary in size from small Golgi-like, smooth-walled vesicles (fig. 16) to large irregular inclusions (fig. 11) closely resembling pigment granules in their internal structure. It appears probable that these vesicles represent early stage of melanin-granule formation (fig. 16). Inside smooth-walled membranous structures, we occasionally observed a delicate filamentous substructure arranged in one direction (fig. 14), which reminds one of Moyer's observation (*20*) suggesting that a developmental sequence in the melanin formation occurs in intracisternal dilatations of the endoplasmic reticulum. In addition to these neoplastic melanocytes, we found pigmented cells containing granules of an entirely different appearance (fig. 15). These granules were composed of relatively large masses containing clusters of pigmented rod-shaped subunits (*14, 19*).

Similar formations have been previously described in macrophages of certain malignant melanomas (*21*, *22*) and presumably represent phagocytized cytoplasmic masses. It is of interest that in previously described tumors these formations were usually unassociated with developmental stages of granule formation and were found in cells that could be identified as macrophages by other criteria. In our material, however, these cytoplasmic masses were found side by side with structures which we interpreted as melanin granules at varying stages of evolution (fig. 15). This suggested that we might be dealing with an unusual type of melanocyte in which formation of melanin granules and phagocytosis take place (*14*). However, since then, we observed striated granules in which accumulations of numerous clusters of melanin rods appear to replace the striated structure (fig. 12). This leads to the formation of pigmented cytoplasmic masses containing numerous clusters of electron-dense material (fig. 15) and resembling those described in macrophages (*21*, *22*). Other interesting characteristics of these tumor cells are the deep indentations of the nuclei and the marked pinocytosis, both features said to be prominent in neoplastic cell in general (*23*).

To compare the melanogenesis in the carcinogen-induced tumors with normal melanogenesis we examined the dermal melanocytes of the golden and white hamsters' ear under the electron microscope (*24*). Grossly the skin in this area is not pigmented at birth but gradually darkens during the first few weeks. Biopsies were taken from the tips of the ears on the 3d, 4th, 7th, 11th, 15th, 21st, 27th, 31st, and 33d day of age. In the white hamster, the earliest evidence of pigmentation of the melanin granules was observed at 21 days. At this age most of the granules were still nonpigmented, but some of them showed evidence of early pigmentation. At 33 days, melanization of most of the granules had progressed to a stage of complete electron density. Similar observations were made in the dermal melanocytes of the golden hamster's ear, except that melanization of the granules started on the 3d day and progressed to complete electron density on the 15th day. Between the white and golden hamster, there was no difference in the substructure of the pigment granules of the normal dermal melanocytes. The fine structure of these granules was similar to that observed in the carcinogen-induced melanotic tumors of the golden hamster. The only difference existing between the normal dermal melanocytes of the hamster's ear and the neoplastic melanocytes of the carcinogen-induced tumors of the golden hamster was the larger size of the latter, 800 mμ versus approximately 500 mμ in transverse diameter. In contrast, the pigmented granules in the carcinogen-induced melanotic tumors of the white hamster showed such pronounced structural aberrations, in comparison with normal melanocytes, that we initially attributed this phenomenon to the effect of the carcinogen upon the precursors of the melanin granules (*14*). However, since identical tumors in the golden hamster show only a slight deviation from the normal morphology, consisting primarily of the larger size of the granules, we have considered the possibility that some differences in the genetic control of melanogenesis between the golden and white variety become apparent through the action of the carcinogen and may account for the bizarre morphologic features of the neoplastic melanocytic granules in the white hamster.

To determine whether an abundance of pre-existing melanocytes predisposes the particular area of the skin to the formation of carcinogen-induced tumors, we applied 400 μg of DMBA in mineral oil to each ear of the golden hamster at the approximate age of 5 to 6 weeks. This, however, failed to produce either melanotic spots or tumors after 48 weeks of observation (*25*). Since the granules of the melanocytes in the adult golden and white hamsters' ears showed a high electron density indicating the complete or almost complete saturation of the granules with melanin, it occurred to us that melanocytes of this type might no longer be susceptible to the carcinogenic effect of DMBA. To test this hypothesis we painted each ear of golden and white hamster, at the age of 14 days, with 50 μg of DMBA. These animals were selected because at that age melanization of the ear melanocytes of the golden hamster was still not completed and in the white hamster had not as yet begun. This experiment is now in the 18th week. As early as the 10th week, melanotic spots began to appear and some have already progressed to tumor formation. Eight-week-old white hamsters were treated under identical conditions, but a dose commensurate with the larger size of the ear and identical with that previously employed in the adult golden hamster was used (400 μg to each ear). No melanotic spots or tumors have appeared with this dose, which is identical with that producing tumors on the skin of the back (*26*). Our findings suggest that nonpigmented dermal melanocytes or dermal melanocytes with nascent pigment granules are susceptible to the carcinogenic effects of DMBA, while melanocytes in which the granules are completely saturated with melanin are resistant to its effect.

There is still some uncertainty as to the precise origin of the perifollicular dermal melanocytes which give rise to the carcinogen-induced melanotic tumors. Three possibilities were considered: 1) that they arise from pre-existing perifollicular melanocytes (*27*); 2) that DMBA causes migration of melanocytes from the hair bulbs into the dermis and such migration is associated with or followed by proliferation of these cells; 3) that they originate from Schwannian cells or from cells of the endoperineurial sheath of dermal nerves as suggested by Masson (*28*) for so-called blue neuronevi.

The smallest melanotic lesions are composed of aggregates of melanocytes in close proximity to the pilosebaceous units (figs. 5 and 6). At that stage they have neither the gross nor microscopic appearance of neoplasms. Some of them undoubtedly proceed to tumor formation. Often the perifollicular localization of the fully developed tumor is readily apparent, particularly when its central portion is included in the sections.

Pertinent to this consistent topographical relationship between hair follicles and melanotic lesions was the discovery of numerous small pigmented spots in whole-skin mounts of normal adult Syrian golden hamster older than 6 months (*27*). Microscopically these spots are also composed of pigmented melanocytes around some pilosebaceous units. We have observed them occasionally in random sections of normal skin of the golden hamster (*14*). Ghadially and Barker (*27*) suggested that the peculiar anatomical arrangement of the melanocytes rendered them vulnerable to the attack by the chemical carcinogen which may enter the hamster skin via the hair follicles and sebaceous glands. They suggested that since the perifollicular network of melanocytes is in places closely applied to the sebaceous gland, direct transfer of carcinogen from sebaceous gland to melanocytes is a distinct possibility (*27*). This would also explain why the hamster is more liable to develop tumors composed of dermal melanocytes than the rabbit and mouse, in which there is no perifollicular network of melanocytes present normally (*27*).

Recently Szabó reported that, in the mouse, hair melanocytes migrate into the dermis during experimental carcinogenesis (*29*). We could not demonstrate such migration of hair melanocytes in sequential biopsies of DMBA-painted skin of the golden hamster. We found occasional pigmented melanocytes in the outer sheath of the hair follicles as early as 1 week following a single application of DMBA, but 2 weeks after such application these melanocytes were no longer demonstrable. They could represent pre-existing cells located in the outer root sheath of the hair follicles and possibly activated by the carcinogen. It was not until the 10th week after painting that we found perifollicular aggregates of melanocytes, which we could not relate to any cells originating in the hair follicles.

There were several histomorphologic features suggesting the possibility that carcinogen-induced melanotic tumors originated from neural elements (*14*). Some of the tumors resembled pigmented neurofibromas (fig. 1). Neurofibrils or entire small nerve fasciculi could be demonstrated in them (fig. 7). In addition pigmented melanocytes were found on occasion in the sheaths of small dermal nerves and in concentric lamellar structures (fig. 8) resembling cutaneous nerve endings located in the proximity of the tumors. Moreover, the abundance of argyrophilic reticulin fibers surrounding virtually every tumor cell (fig. 3) is in striking contrast to the paucity of reticulin fibers in the malignant melanoma (fig. 4) of man and hamster. It reminds one of Masson's description of so-called blue neuronevi, in which the melanocytes are believed to originate from either Schwannian cells (*28*) or cells of the endoperineurial sheath (*28, 30*), and have retained the capacity of the neuroectodermal mesenchyme to form reticulin and collagen. Because of this we have paid special attention to finding neurogenic components in the tumor tissue under the electron microscope. In fairly large tumors, neural structures could be rarely observed. However, when we proceeded to examine the early stages of melanotic tumor formation in either golden or white hamster, many myelinated and nonmyelinated nerve fibers enveloped by Schwannian cells were present among the tumor cells (*31*). They were most abundant in close proximity to the hair follicles. Some of the myelinated nerve fibers located more distantly from the hair follicles showed degenerative changes (fig. 19), possibly due to the mechanical compression by the growing tumor tissue. This degeneration appeared to occur primarily in axons, in the form of swollen and irregularly arranged axon filaments and increased electron density of the axoplasm (fig. 19). At the same time mitochondria and tubules were no longer observable. At a later stage of degeneration of the axons, the arrangement of myelin became irregular. This was associated with atrophic and degenerative changes of Schwannian cells. Finally, the axons together with Schwannian cells progressed to complete degeneration resulting in the formation of almost amorphous masses (fig. 19) that were frequently observed at a late stage of melanotic tumor formation (*31*). This sequence of events explains why we could not demonstrate, in studies limited to the light microscope, any significant increase of nerve elements even in those carcinogen-induced melanotic tumors which resembled neurofibromas (*14*). The hypothesis of the neurogenic origin of the carcinogen-induced melanotic tumors in the hamster received further support from an electron microscopic finding which essentially confirmed one of our previous observations (*14*), namely, the presence of pigmented melanocytes within cutaneous nerves in hamsters painted with DMBA. In one of the smaller melanotic tumors of the white hamster, we found melanocytes entirely located within the perineurium of a small nerve fascicle [figs. 17 and 18, (*31*)]. The melanin granules had the characteristic striated substructure and contained some rod-shaped electron-dense bodies (figs. 17 and 18) previously observed in neoplastic melanocytes in the white hamster. Since these cells were devoid of mesaxons we tentatively interpreted them as pigmented endoneurial sheath cells rather than Schwannian cells, even though, according to some observers, a large proportion of so-called endoneurial fibroblasts are probably Schwannian cells (*32*). This finding demonstrates that cells of the neuroectodermal mesenchyme of the Syrian hamster are susceptible to the melanogenetic effects of the chemical carcinogen DMBA and also suggests the possible origin of the carcinogen-induced melanotic tumors of the hamster from cellular components of cutaneous nerves (*31*). This observation would also account for the fact that the tumor cells, being derived from neuroectodermal supportive tissue cells of peripheral nerves are capable of forming reticulin and collagen in addition to melanin. It could also explain the perifollicular localization of the early lesion since there is a particularly abundant network of delicate nerve fibrils around the hair follicles (*33*). It would suggest that the carcinogen-induced melanotic tumors may not necessarily arise from pre-existing perifollicular networks of melanocytes as postulated by Ghadially and Barker (*27*), but that nonpigmented Schwannian cells of the perifollicular network of nerve fibrils are equally susceptible to the melanogenetic and carcinogenic effects of DMBA. It is also likely that the perifollicular dermal melanocytes found in hamsters beyond the age of 6 months (*27*) are derived from Schwannian elements and that they appear in the normally nonpigmented dermis in response to nonspecific stimuli as the animal becomes older. The induction of melanotic spots by long-term, repeated applications of croton oil (*18*) appears to be consistent with this interpretation.

The rarity of spontaneous malignant melanoma in the Syrian golden hamster and the absence of this neoplasm in our series of DMBA-treated animals prompted us to investigate, in this species, certain morphologic and biologic aspects of the epidermal melanocyte, presumed to be the cell of origin of malignant melanoma. Even though we could not demonstrate pigmented or nonpigmented dopa-positive melanocytes in the normal epidermis in either the golden or white Syrian hamster under the light microscope, circumstantial evidence appeared to indicate that such cells exist. First, we found pigmented epidermal melanocytes in 2 benign papillomas, each of which occurred in a DMBA-treated golden hamster bearing melanotic tumors. Secondly, the occurrence of malignant melanomas resembling the corresponding tumors in man would indicate the existence of epidermal melanocytes. However, it was not until we examined the epidermis under the electron microscope that we found relatively small numbers of clear cells devoid of desomosomes and tonofilaments (*14*, *20*). Each contained an indented nucleus, a prominent Golgi zone, and some organelles, racquet- or rod-shaped in profile, resembling those recently described in basal melanocytes and high-level clear cells of patients with vitiligo (*34*). These clear cells could not be converted into dopa-positive cells either by irradiation with ultraviolet light (1 erythema dose daily for 7 consecutive days) or by single cutaneous application of DMBA (approximately 800 μg of DMBA). However, following either treatment in the white hamster, dopa reactivity of the melanocytes in the hair bulb at a stage of anagen was very much increased in comparison with the weak dopa positivity of the hair bulb melanocytes at the same stage of hair cycle in the untreated animal (*14*). We tentatively concluded that the apparent lack of susceptibility of the inactive epidermal melanocytes of the Syrian hamster to the melanogenic and carcinogenic effect of DMBA may account for the absence of malignant melanomas in our DMBA-treated animals.

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PLATES

PLATE 25

FIGURE 1.—Poorly pigmented DMBA-induced melanotic tumor of white hamster, composed mostly of nonpigmented spindle-shaped cells. *Note* characteristic cellular arrangement closely resembling neurofibroma. Hematoxylin and eosin. Reprinted from *Cancer Research;* Rappaport (*15*). × 150

FIGURE 2.—Malignant melanoma of golden hamster. Part of dermoepidermal junction is involved by tumor. Tumor cells are round or oval to polygonal in shape and subdivided in groups by delicate strands of connective tissue. Hematoxylin and eosin. Reprinted from the *Annals of the New York Academy of Sciences;* Rappaport (*14*). × 275

FIGURE 3.—Poorly pigmented DMBA-induced melanotic tumor of white hamster. Abundance of argyrophilic reticulin fibers enveloping virtually every tumor cell is in striking contrast to reticulin pattern of malignant melanoma illustrated in figure 4. Snook's modification of the Maresch-Bielschowsky stain. Reprinted from *Cancer Research;* Rappaport (*15*). × 100

FIGURE 4.—Malignant melanoma of golden hamster showing alveolar reticulin pattern surrounding groups of many tumor cells. Snook's modification of the Maresch-Bielschowsky stain. Reprinted from the *Annals of the New York Academy of Sciences;* Rappaport (*14*). × 100

PLATE 26

FIGURE 5.—A small DMBA-induced melanotic lesion of golden hamster. Numerous heavily pigmented dermal melanocytes proliferate in close proximity of a hair follicle. Hematoxylin and eosin. Reprinted from *Cancer Research;* Rappaport (*15*). × 150

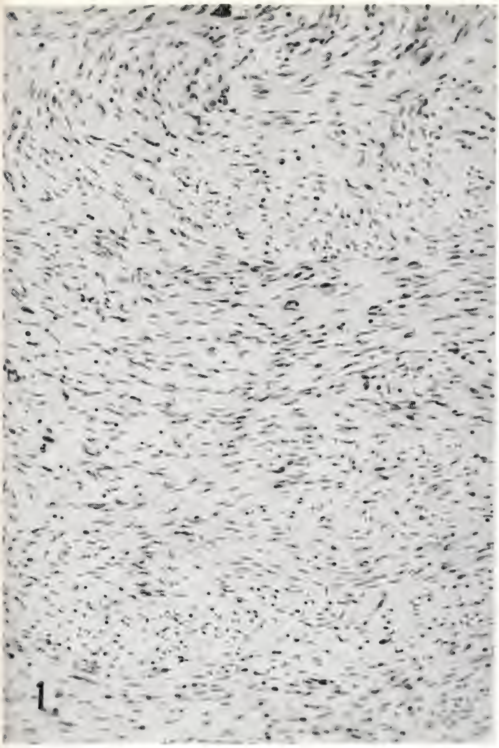
FIGURE 6.—A small DMBA-induced, poorly pigmented melanotic lesion of white hamster. *Note* perifollicular proliferation of amelanotic melanocytes. Hematoxylin and eosin. × 100

FIGURE 7.—A small nerve fascicle and a few delicate nerve fibers surrounded by pigmented and nonpigmented tumor cells in DMBA-induced melanotic tumor of white hamster. Spindle-shaped nuclei adjacent to some nerve fibers possibly belong to Schwannian cells. Bodian's method for nerve fibers. Reprinted from the *Annals of the New York Academy of Sciences;* Rappaport (*14*). × 375

FIGURE 8.—A neural structure in vicinity of DMBA-induced melanotic tumor of golden hamster, showing a concentric lamellar arrangement resembling a cutaneous nerve ending. *Note* pigmented melanocytes along *margin* and *inside* of structure. Hematoxylin and eosin. × 225

PLATE 27

FIGURE 9.—Electron micrograph of DMBA-induced melanotic tumor of golden hamster, showing portions of tumor cells located adjacent to hair follicle. Nonpigmented cell with desmosomes in *upper left corner* is a hair follicle cell. Tumor cells contain numerous melanin granules in cytoplasm. *Note* abundance of connective-tissue fibers between cell borders consistent with the histologic observation of the reticulum pattern of this tumor. × 7,500



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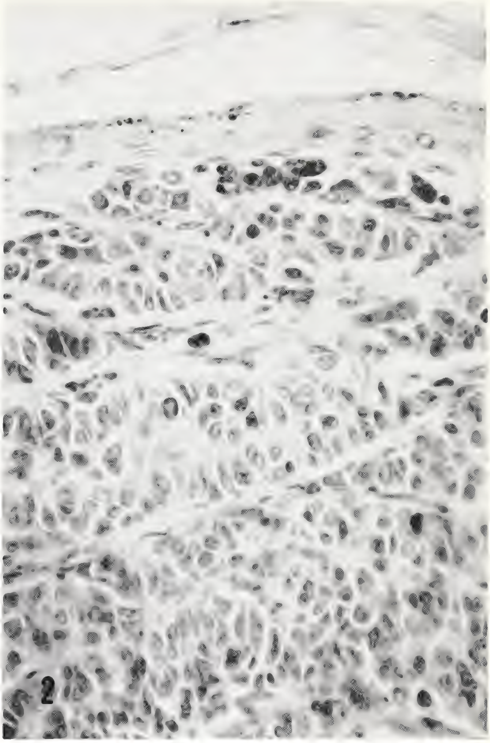

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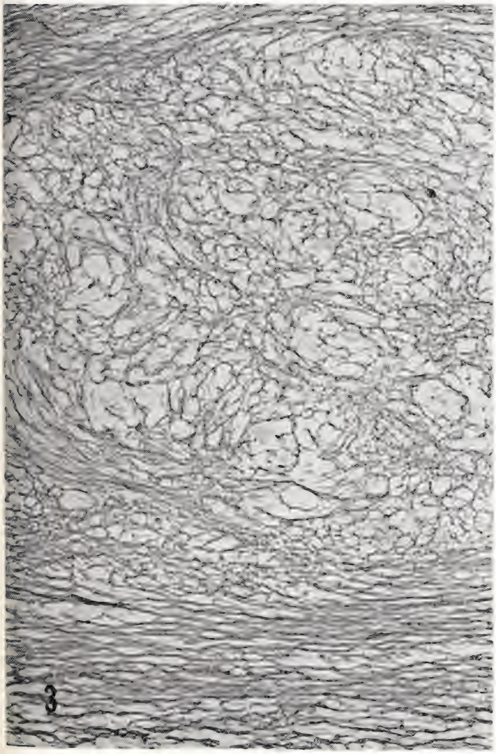

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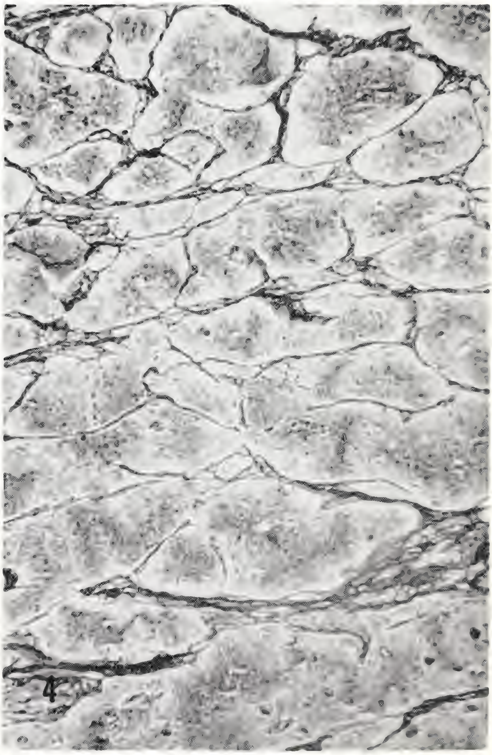

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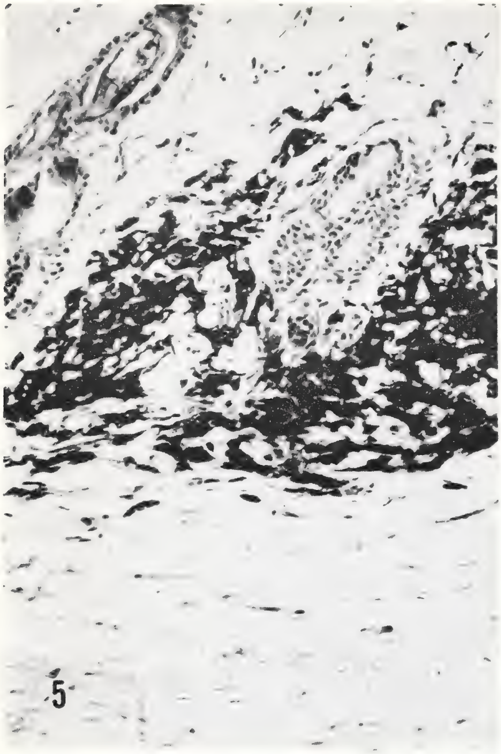

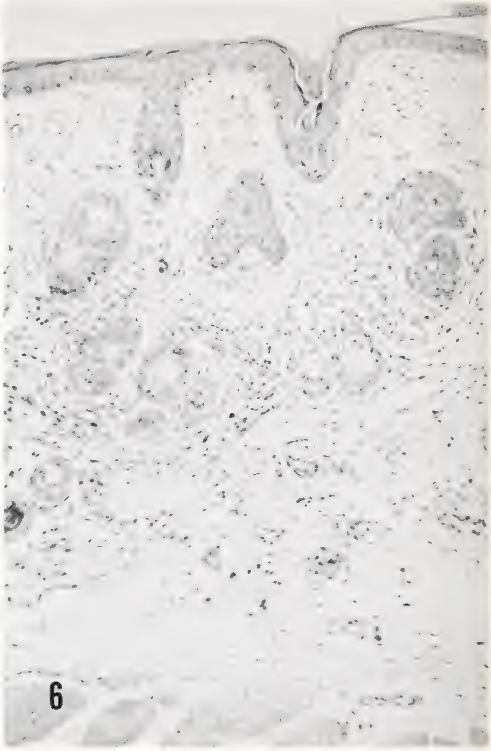

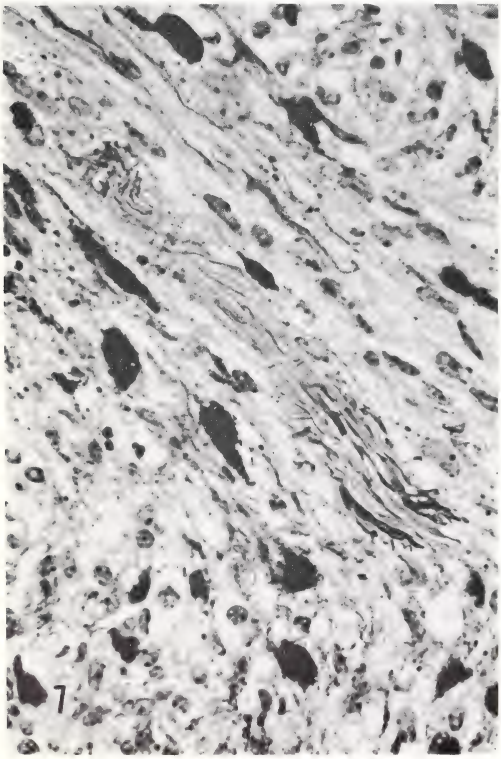

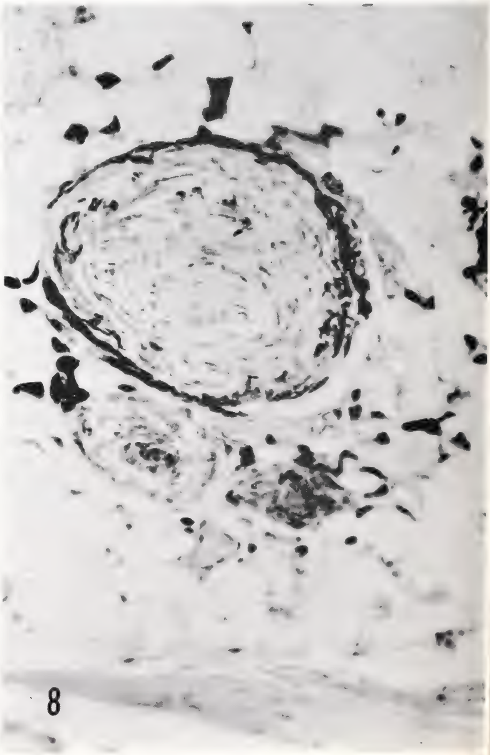

NAKAI AND RAPPAPORT

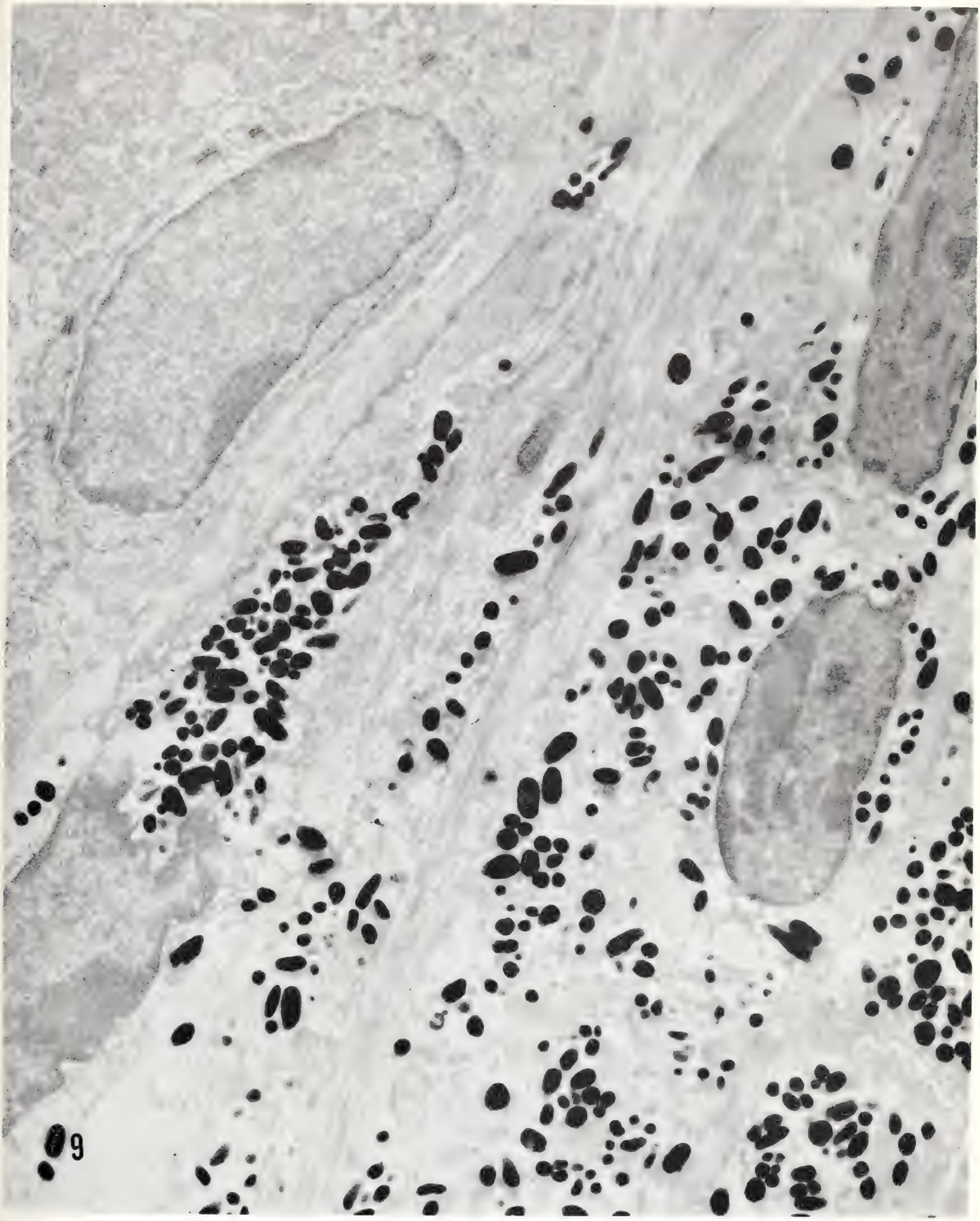
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PLATE 28

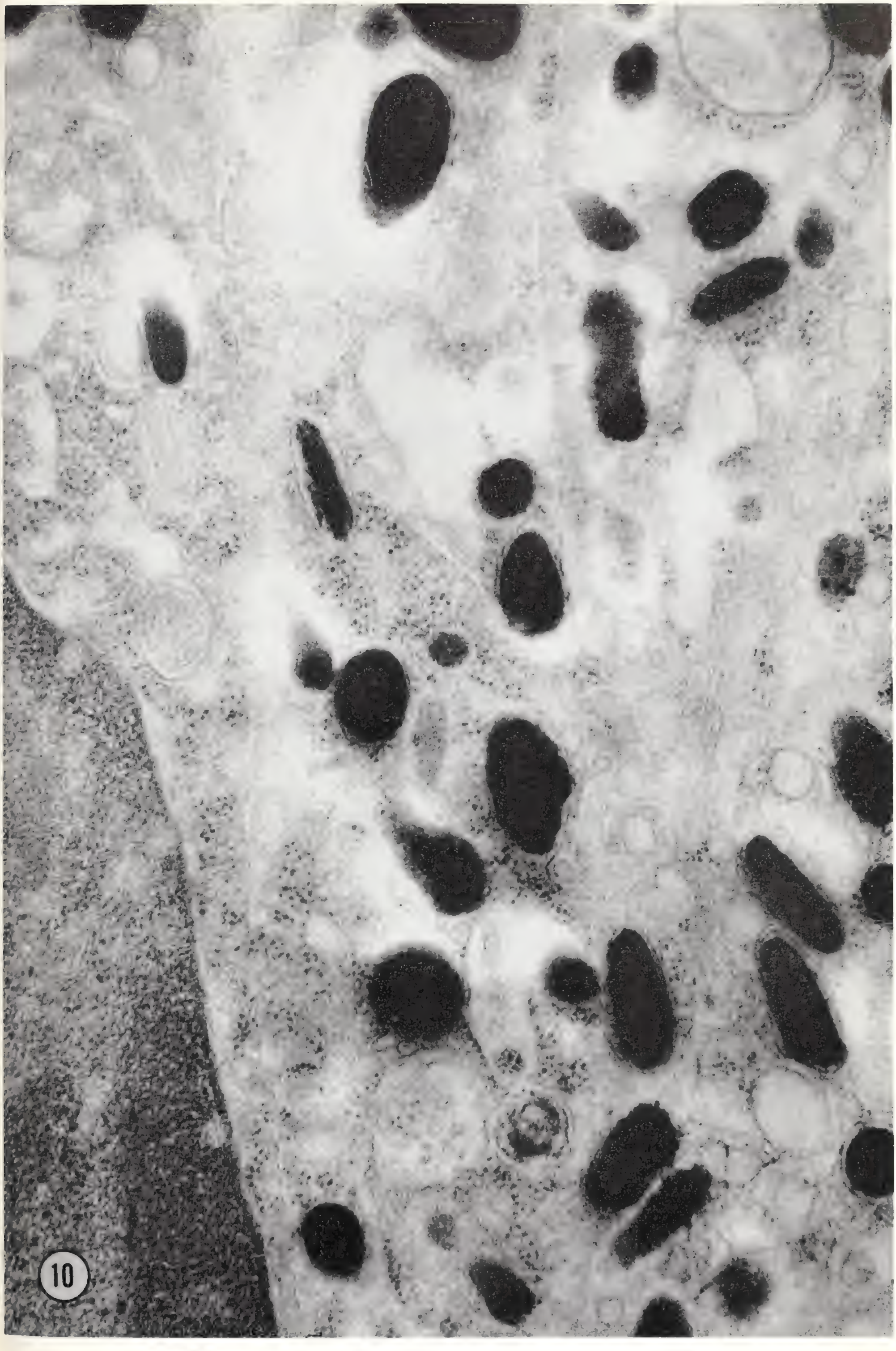
FIGURE 10.—Portion of tumor cell of small DMBA-induced melanotic tumor of golden hamster, containing numerous pigment granules at various stages of evolution. Many granules have a high electron density and are surrounded by a single smooth membrane. Near *left margin* of illustration and close to upper pole of the nucleus are two nonmelanized pigment granules, one cut longitudinally in which oriented fibrillar structures with a distinct periodicity can be discerned, the other obliquely. *Note* scattering of smooth-walled vesicles, some resembling precursors of pigment granules. × 41,500

PLATE 29

FIGURE 11.—Electron micrograph of DMBA-induced melanotic tumor of white hamster, illustrating portions of 2 tumor cells, 1 containing numerous pigment granules. Connective-tissue fibers are seen between cells. × 16,000

PLATE 30

FIGURE 12.—Same material as in figure 11. Pigment granules show great variations in size and shape. All possess a striated internal structure; in some granules electron-dense rods representing melanin are seen along striations. Some portions of striated material are replaced by clusters of these melanin rods. *Compare* atypical structures with orderly morphologic evolution of melanin granules in figure 10. × 37,000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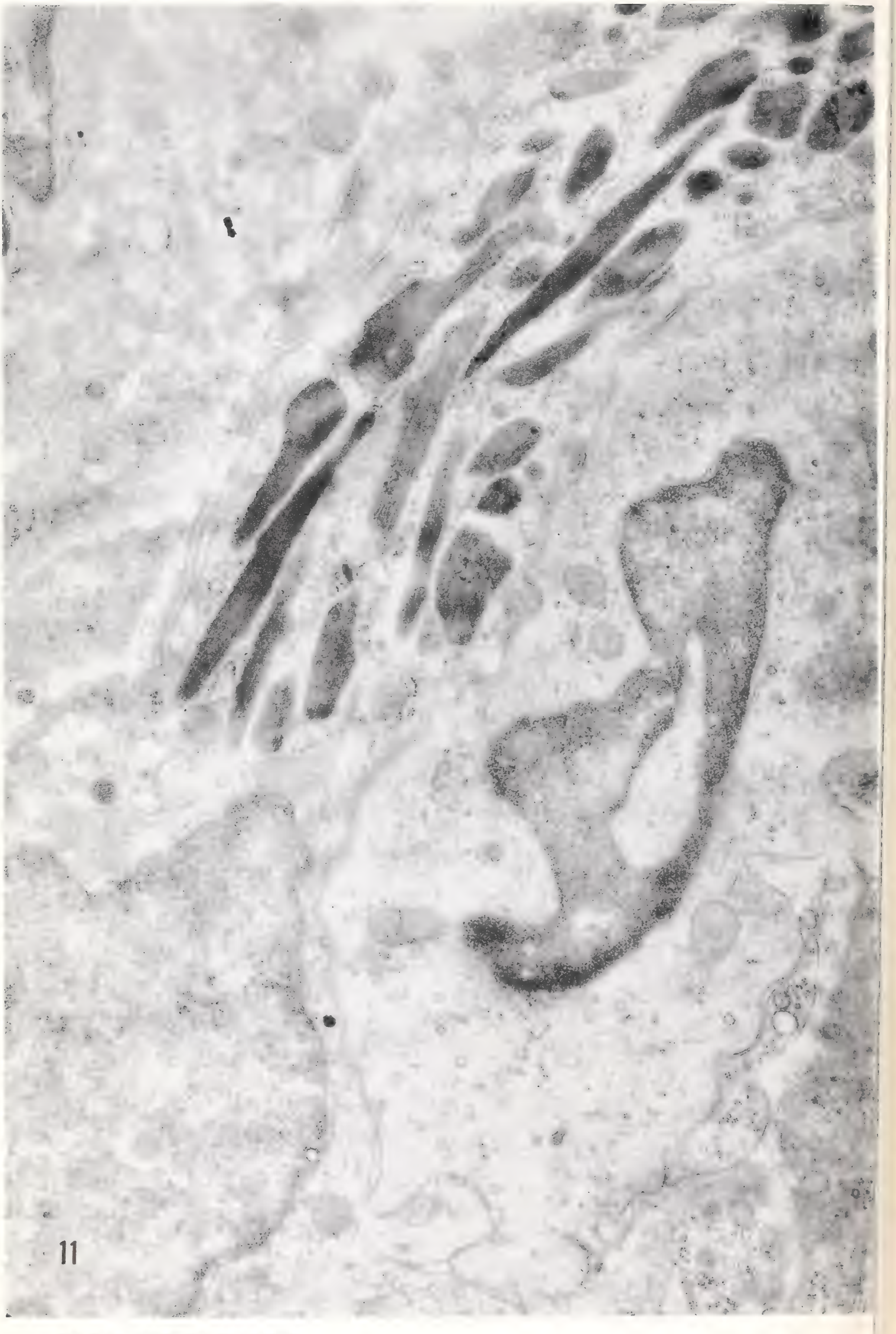

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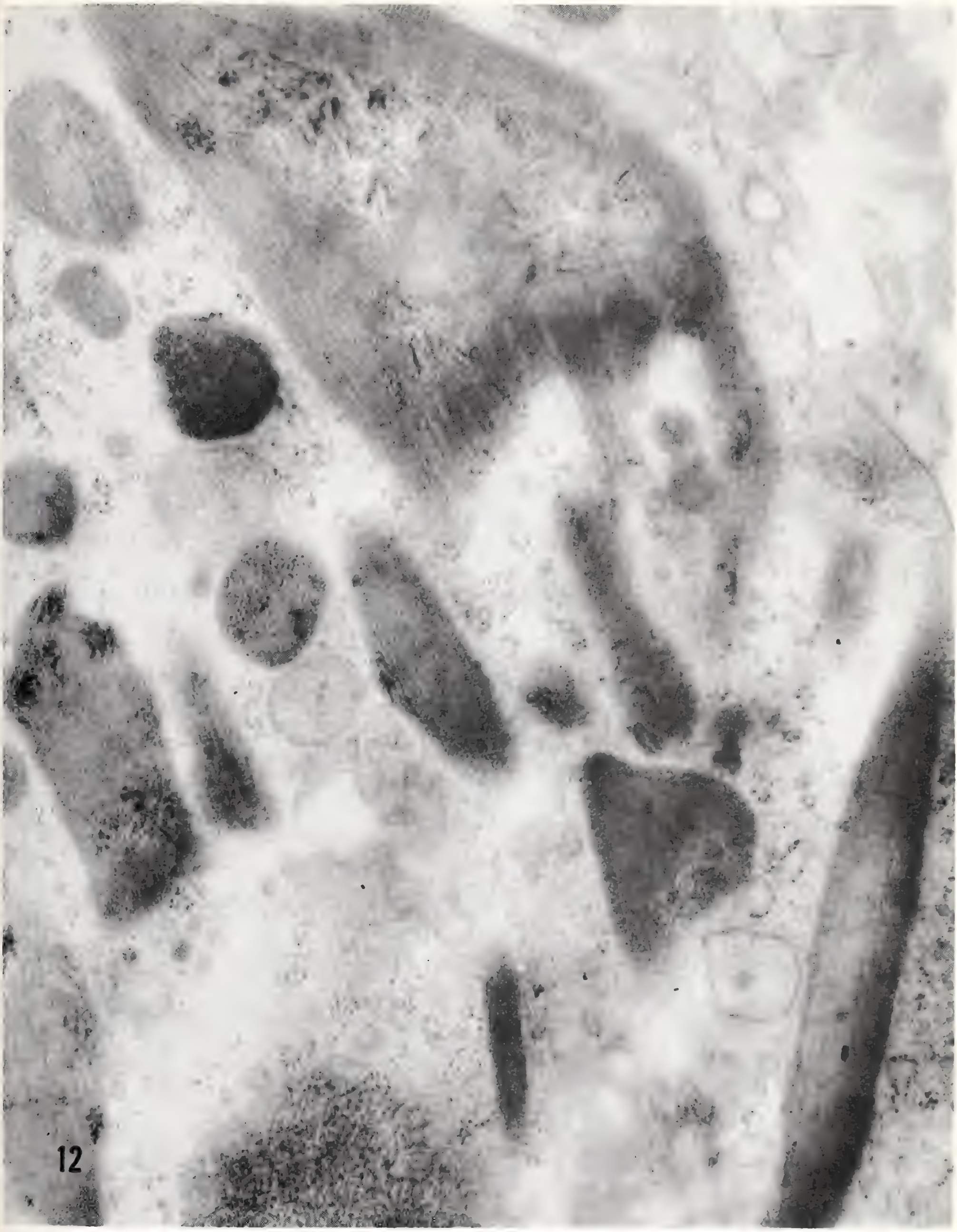
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PLATE 31

FIGURE 13.—Same material as in figure 11. Cross sections of 2 striated pigment granules illustrated at *top left*. One adjacent to *upper margin* shows a crystalloid substructure while one *below* it and to *left* has granular appearance, with the granules representing cross sections of striae. × 32,000

FIGURE 14.—Same material as in figure 11. Portion of a tumor cell containing early precursors of pigment granules, characterized by a crystalloid filamentous substructure and surrounded by smooth-walled membranes. *Note* granule, near *right margin,* with 2 crystalloid formations arranged in different directions. × 42,500

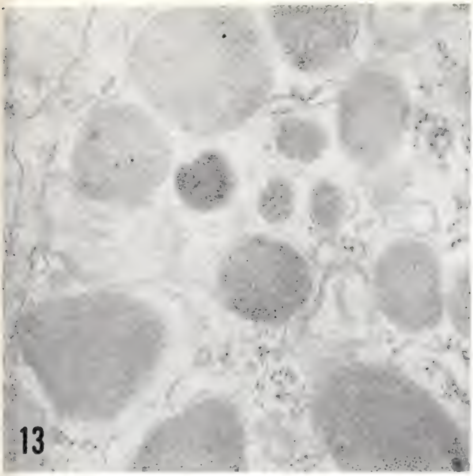
FIGURE 15.—Same material as in figure 11. Larger granules contain clusters of melanin rods, surrounded by granular material of lower electron density, located side by side with smaller granules possessing striated material but devoid of melanin rods. × 23,500

PLATE 32

FIGURE 16.—Same material as in figure 11. Cytoplasmic process of nonpigmented tumor cell, showing centrioles, several prominent Golgi zones, and numerous pigment granules of varying electron density. Larger and elongated granules have striated substructure. × 32,000

PLATE 33

FIGURE 17.—Electron micrograph of small DMBA-induced melanotic tumor of white hamster showing cross section of a small nerve fascicle. *Note* that portion of cell within nerve fascicle contains a number of characteristic striated granules interpreted as amelanotic precursors of melanin granules. This cell is unassociated with myelin and devoid of a mesaxon. Nonpigmented tumor cell outside of perineurium is seen at *bottom* of illustration. × 7,500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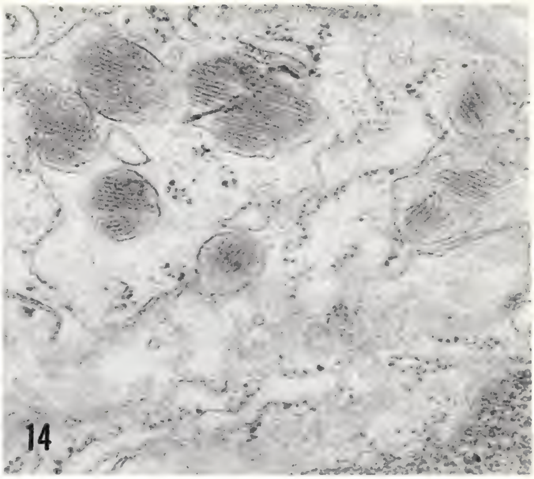
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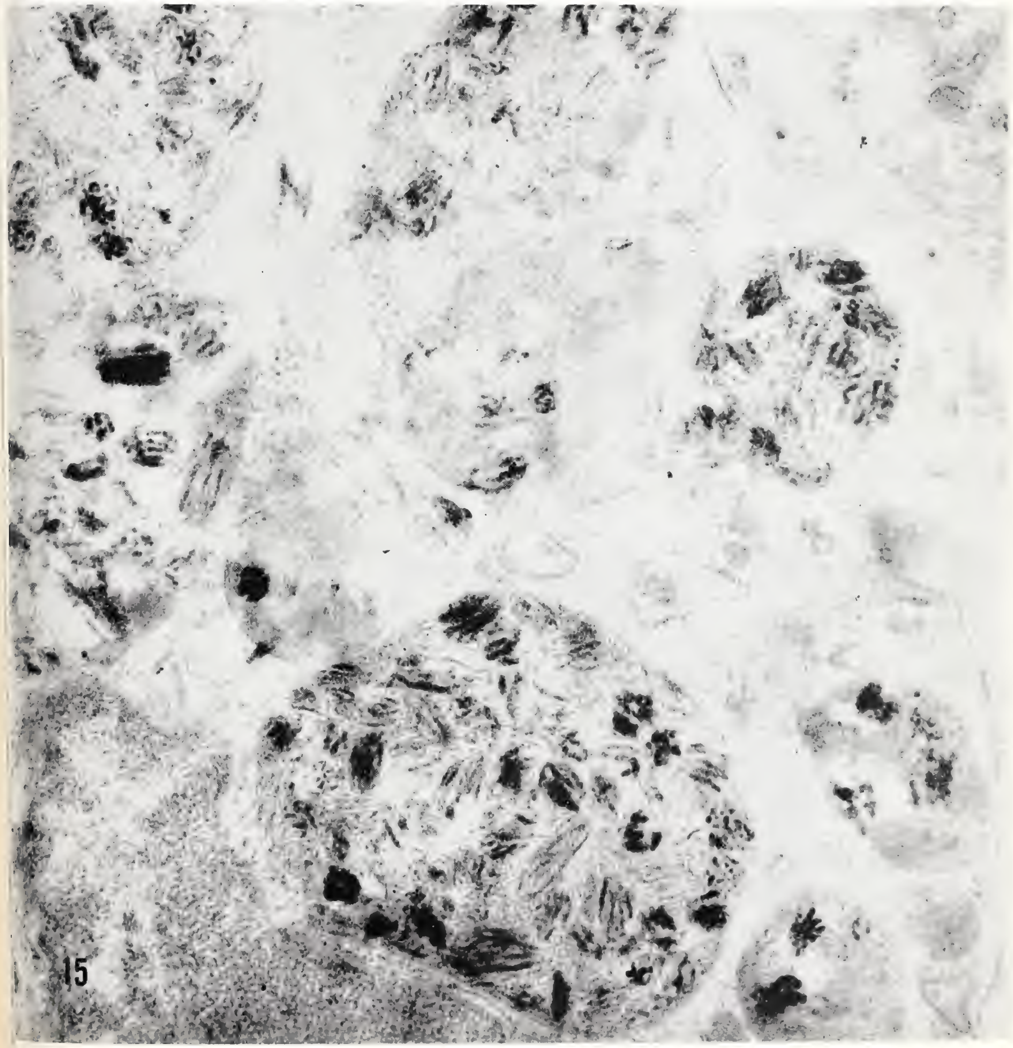
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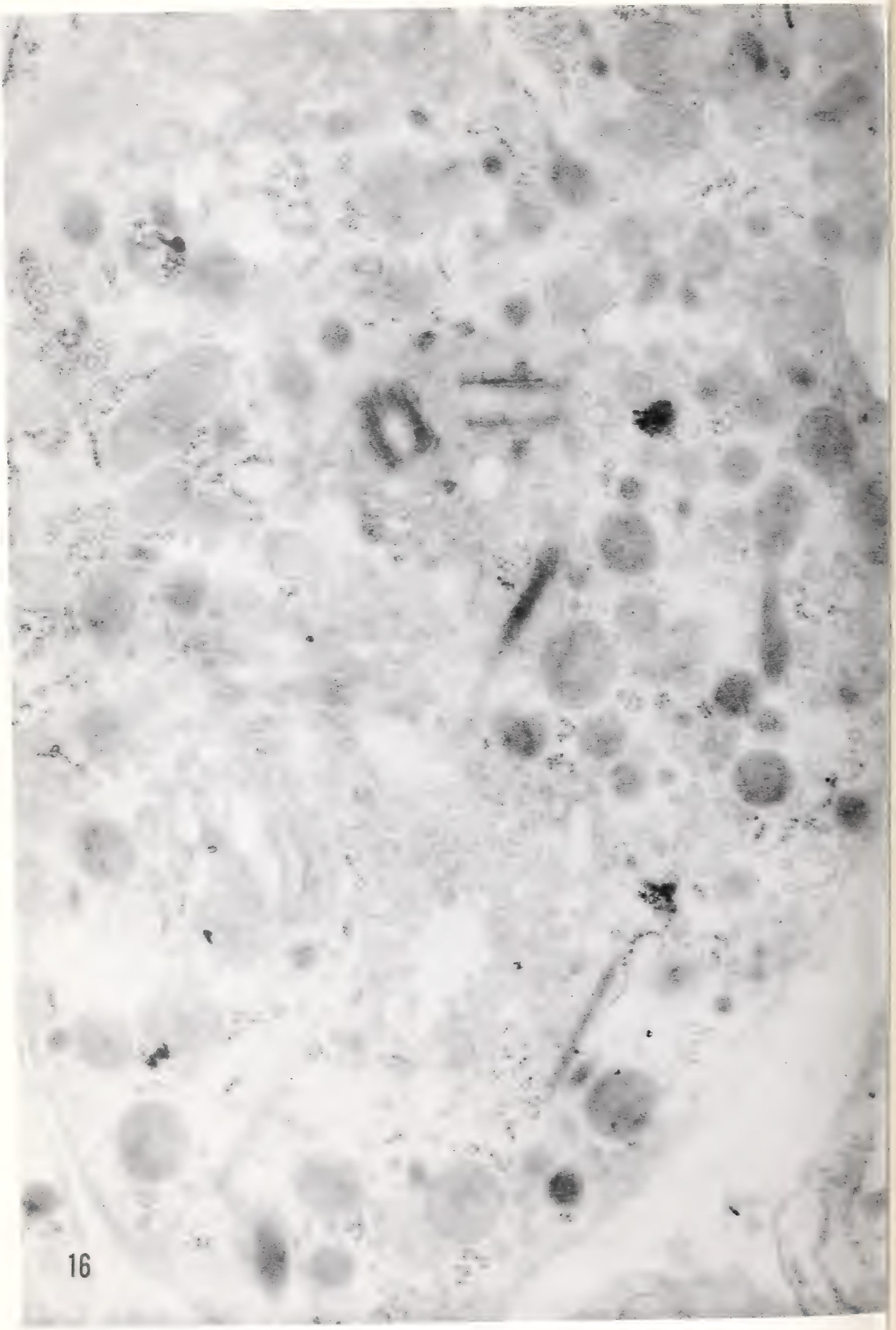

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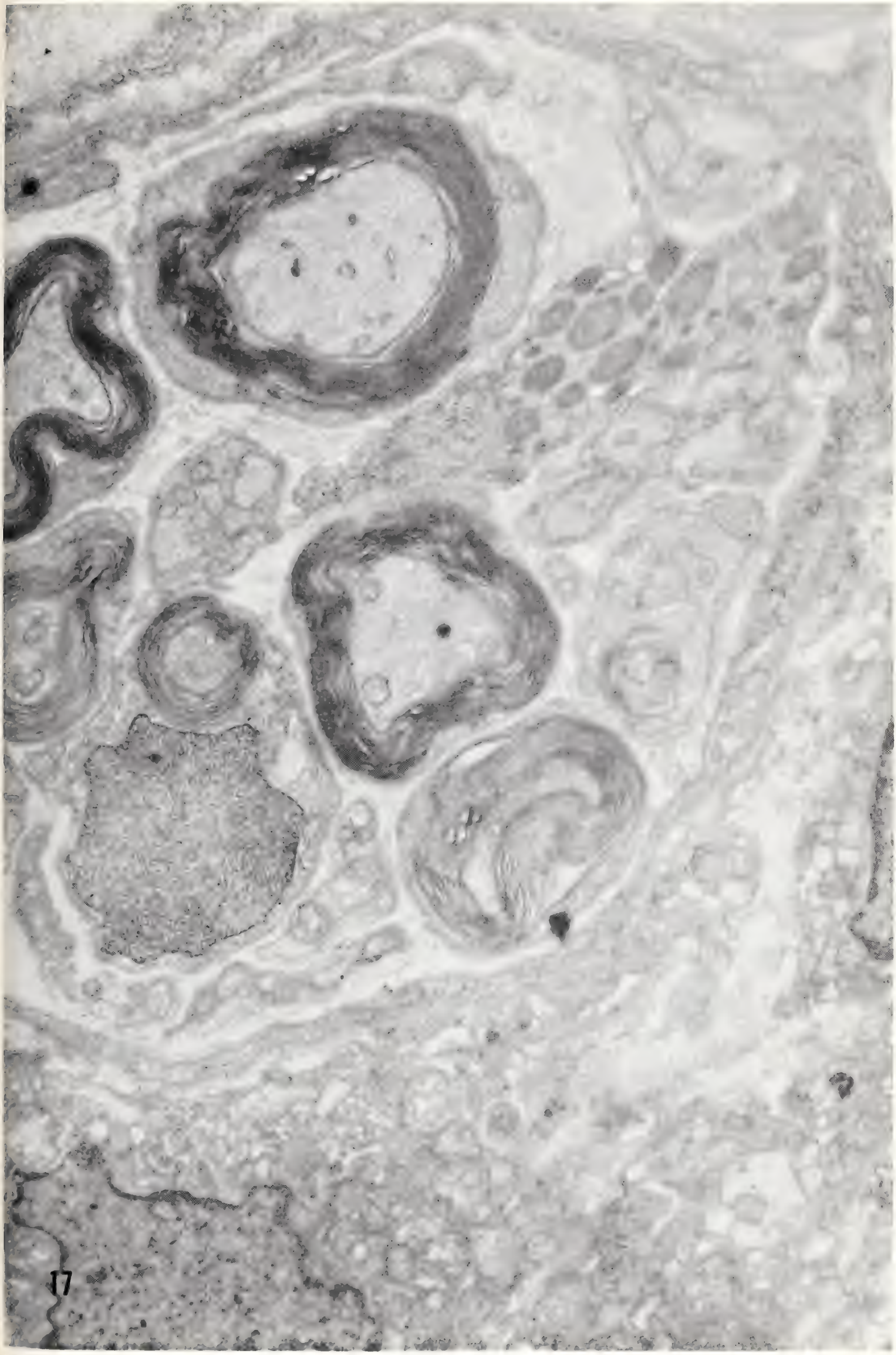
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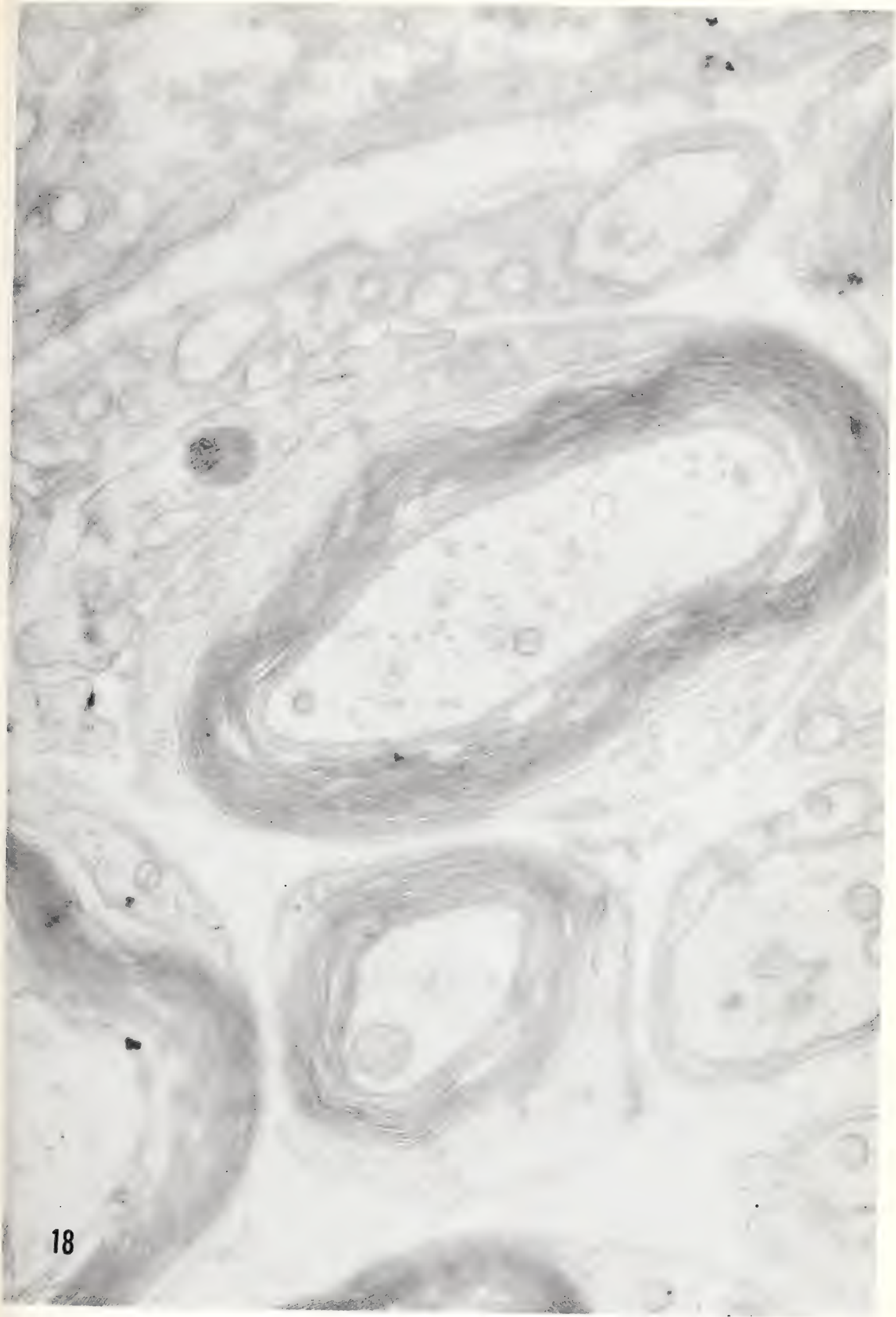
PLATE 34

FIGURE 18.—Same material as in figure 15. Higher magnification shows characteristic pigment granule composed of striated material and melanin rods in cytoplasmic process of endoneurial sheath cell. Portion of a perineurial sheath cell is illustrated at *top*. × 23,000

PLATE 35

FIGURE 19.—Same material as in figure 15. Three degenerating myelinated nerve fibrils surrounded by portions of tumor cells. Electron-dense structure at *top* is interpreted as cross section of a myelinated nerve fibril in state of advanced degeneration. × 7,500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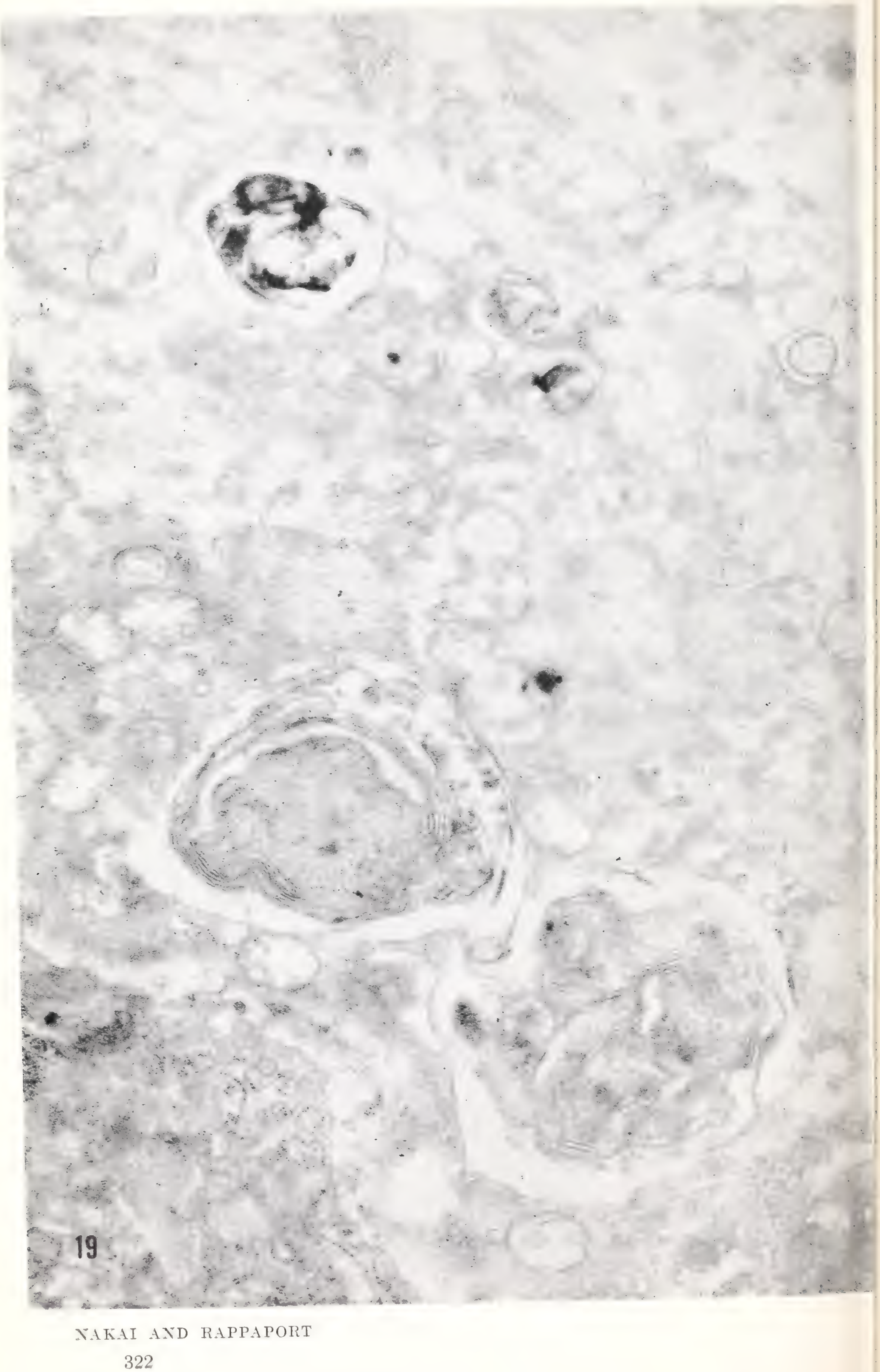

NAKAI AND RAPPAPORT

CUTANEOUS PRECANCEROUS CONDITIONS IN MAN[1, 2]

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SUMMARY

This report presents recent concepts and observations of the cutaneous precancerous conditions of Bowen's disease, arsenical keratosis, erythroplasia of Queyrat, senile keratosis, extramammary Paget's disease, and intraepidermal epithelioma of Jadassohn. The association of Bowen's disease and arsenical keratosis with cutaneous, extracutaneous, and internal cancer is shown. Because of the similarities of Bowen's disease and arsenical keratosis and their association with systemic cancer, it is probable that arsenic represents a common etiologic agent. Even though there is a striking microscopic resemblance of erythroplasia of Queyrat to Bowen's disease, the natural history indicates that erythroplasia of Queyrat is a different disease. The histopathologic features of senile keratosis may at times be confused with Bowen's disease and preliminary data suggest that they may behave biologically similarly with regard to systemic cancer. Squamous-cell carcinoma will occur in at least 12 percent of patients with untreated lesions of senile keratosis, but the natural history indicates that this lesion rarely if ever metastasizes. This course is completely different from that seen in the primary or *de novo* type of squamous-cell carcinoma which is an aggressive lesion. Extramammary Paget's disease of the anogenital region is a distinct clinicopathologic entity. At least half of the patients have an associated adnexal skin cancer or primary cancer of the rectum, urethra, or breast. Intraepidermal epithelioma of Jadassohn may be confused with Bowen's disease but the lack of association with systemic cancer is of striking statistical significance. It is a distinct clinicopathologic lesion and, based on the occurrence of invasion of the corium in 8 percent of the patients and metastasis in one instance, is classified along with the other precancerous dermatoses.—NCI Monogr. No. 10: 323–333, 1963.

THE TENDENCY of cancer to develop in a certain group of skin lesions has for many years been recognized and has given rise to the term "precancerous dermatoses." While this term cannot be recommended as the final one, it is acceptable until a more scientific and exact classification can be devised. The purpose of this report is to call attention to recent

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

[2] This work was made possible in part by grant C-4275 from the National Cancer Institute, National Institutes of Health, Public Health Service.

concepts and observations as a result of our study of the cutaneous precancerous conditions of Bowen's disease, arsenical keratosis, erythroplasia of Queyrat, senile keratosis, extramammary Paget's disease, and intraepidermal epithelioma of Jadassohn.

BOWEN'S DISEASE

In 1959 and 1961 we (*1, 2*) reported a previously unrecognized association of Bowen's disease with primary systemic cancer. Since our initial report (*1*), evidence published by others (*3–5*) supports this new concept. In this report we shall record our additional experience and observations of patients with Bowen's disease.

Anatomically, 212 lesions from 155 patients were studied histologically and these were single (65%) or multiple (35%) and occurred about equally on exposed and unexposed areas of the body. Clinically, the lesions appeared as lenticular, polycyclic, erythematous, pigmented, crusty, scaly, keratotic, nodular, and ulcerated plaques, all sharply demarcated from the surrounding tissue. The median diameter was 1.3 cm. The initial clinical diagnosis was most frequently some variety of carcinoma, benign neoplasm, or inflammatory process. The disease afflicted predominantly the older age groups. The average age at onset was 48.4 years and at the time of first biopsy, 55.1 years. The average duration from onset to removal was 6.4 years. The lesions occurred in both sexes and predominantly in Caucasian males with combinations of light complexion, hair, and eyes, who had spent most of their lives in the southern United States. The lesions also occurred in Negro, Oriental, and Latin American patients.

Excision of the Bowen's lesion was the treatment of choice since the process tended to be resistant to X-ray therapy, and prone to recur after desiccation and curettement. Eighty percent of all patients who have Bowen's lesions excised can expect to live for 15 years, or longer, without having a recurrence. Of those patients who have recurrences, 50 percent can be expected to occur within 10 months after excision. Adequate treatment of the Bowen's lesion prevents a larger number from invading the deeper stroma and from subsequent metastasis to internal structures, but treatment has no definite preventive effect on the subsequent occurrence of other premalignant and malignant cutaneous lesions, extracutaneous cancer, and internal malignant neoplasms.

On an average of 6 to 7 years after the onset of Bowen's disease, 64 (41.3%) of the group had a total of 238 premalignant and malignant lesions of the skin, which included senile keratosis, basal-cell carcinoma, metatypical basal-cell carcinoma, multicentric basal-cell carcinoma, squamous-cell carcinoma, senile keratosis with squamous-cell carcinoma, adenoacanthoma, adnexal carcinoma, melanoma, sweat-gland carcinoma, and adenocystic carcinoma. These occurred in 25 different combinations. Two patients with primary squamous-cell carcinoma and 1 with malignant melanoma evidenced metastasis to internal structures.

One fourth of the entire group had primary internal or extracutaneous cancers that were detected at an average of 5½ years after the onset of Bowen's disease. Pathologic examination showed primary internal or extracutaneous (lip, eye, oral cavity, breast) cancer in 32, or 70 percent, of 46 patients who died; 29 of these 32 patients also had metastasis. The internal malignant tumors in 8 patients were first detected at autopsy. Eight patients had multiple combinations of primary systemic cancers.

Median survival time for the entire group was 12 years after the diagnosis of Bowen's disease was established, and 4.6 years for patients with Bowen's disease and internal cancer in the age group 40 years and over. An abridged life table was constructed to calculate the probability of detection of the first primary internal cancer in males at a specific age. Follow-up reports, surgical specimens, or autopsy material received subsequent to removal of Bowen's lesions in 127 males of known ages, cover a span of 15 years (1942–57). No primary internal cancers were found before age 40; thus, the calculated cumulative probability is that 65.82 per 100 males with Bowen's lesions would have primary internal cancer at or after age 41. This probability value does not differ significantly at the 1 percent level from any population values between 57.29 and 74.35 percent. The risk of developing such a cancer, or rather the probability that it would be detected, increases with age from 0.87 per 100 at age 41 to 22.0 at age 86. A similar life table for the years 1949–51, with use of ages in 1950 of the survivors without cancer revealed that 19.67 percent would be found at age 45 or later; 3 of the 9 internal cancers detected during this period were in the stomach, and single sites were lung, bladder, mouth, pharynx, eye, and reticuloendothelial system. Additional sites of a total of 36 internal or extracutaneous cancers among males were the intestine, rectum, prostate, kidney, esophagus, larynx, and reticuloendothelial system.

Information was obtained from 77 patients with Bowen's disease regarding malignant tumors in their immediate relatives. One or more members of 37 families (48%) had cancer. History from 105 patients with Bowen's disease suggested exposure to physical elements, insecticides, sprays, dusting powders, trauma, petroleum by-products, drugs, arsenic, and the presence of pre-existing lesions as possible etiologic factors.

The initial pathologic diagnoses made by others were most frequently those of Bowen's disease, squamous-cell carcinoma, squamous-cell carcinoma *in situ*, senile keratosis, intraepidermal carcinoma, basal-cell carcinoma, Paget's disease, and a variety of benign neoplasms and inflammatory processes. Microscopic examination of paraffin block hematoxylin- and eosin-stained sections of a typical Bowen's lesion showed the following: Under the low-power objective there was a plaquelike lesion with hyperkeratosis, parakeratosis, acanthosis, and an inflammatory infiltrate in the upper corium. Under higher power, there was loss of normal architecture and cellular atypism in the form of multinucleated cells, vacuolated cells, dyskeratosis, and abnormal mitotic figures. These changes were confined by an intact basement membrane and were present at all levels of the epidermis. The inflammatory infiltrate in the upper corium was composed predominantly of lymphocytes, histiocytes, and plasma cells. Examination of multiple sections from the same lesion will invariably show involvement of the pilosebaceous follicle, lack of involvement of the epidermal eccrine sweat-duct unit, and areas of uninvolved epidermis. In 8 patients (5.2%) of the 155, the Bowen's process invaded the corium as carcinoma; in 3 (37.5%) of the 8 there was deep invasion and all had microscopic and gross pathologic evidence of metastasis to internal organs. The cytological features of the metastatic lesions appear similar to the atypical epithelial proliferation within the epidermis and corium.

In summary, the data support our initial report that Bowen's disease of the skin represents a cutaneous sign of cancer proneness and is associated with cutaneous, extracutaneous, and internal cancer. Cancer involved all 3 germ layers. The sites in order of frequency were the skin, respiratory system, gastrointestinal tract, genitourinary organs, reticuloendothelial system, endocrine system, breast, and eye. Cancer proneness of the entire group is evident when it is considered that 75 (48.4%) of all the patients had premalignant lesions and/or primary cancer in one or more locations. Probable causes are hereditary predisposition and chemical and physical carcinogens. The association of Bowen's disease of the skin with cutaneous, extracutaneous, and internal cancer may well represent a cutaneous manifestation of a systemic carcinogenic disease process.

Further follow-up of 99 patients living at last report is in progress.

ARSENICAL KERATOSIS

The association of arsenical keratosis of the skin with cutaneous and internal cancer is accepted and has been recognized by most authorities for many years. The recent report by Roth (*6*) summarizes his experiences and supports the observations of others.

In 1959 we (*1*) reviewed material from 15 patients with keratosis and a definite history of ingestion or contact with arsenic, such as Fowler's solution, arsenate of lead, arsenious acid, and other injectable and oral compounds. Anatomically, 44 lesions from the 15 patients were studied histologically, and these were single (53%) or multiple (47%) and occurred on both exposed and unexposed areas of the body. The average age at the time of first biopsy was 49 years, the average duration from onset to removal was 8 years. The lesions occurred in 13 males and 2 females and all were Caucasian with characteristics similar to those seen in patients with Bowen's disease. Clinically and histologically these arsenical keratoses were practically indistinguishable from those of Bowen's disease. Furthermore, a comparison of the natural history of patients with Bowen's disease and those with prolonged contact or treatment with inorganic arsenicals showed a striking similarity. Eight patients (53%) of the group had histologic evidence of premalignant and malignant cutaneous lesions which included senile keratosis, squamous-cell carcinoma, basal-cell carcinoma, and multicentric basal-cell carcinoma. In 1 patient (6.7%) there was histologic evidence of arsenical keratosis with invasion of the corium as carcinoma. In 3 patients (20%) with squamous-cell carcinoma, there was metastasis to internal organs. Two patients (13%) had internal cancer. Five patients are dead—3 as a result of their metastasizing cutaneous or internal carcinomas. Cancer proneness of the group is evident when it is considered that 10 (66%) of the patients had premalignant lesions and/or primary cancer in one or more locations.

Because of the striking similarities of Bowen's disease and arsenical keratosis, we (*7*) performed analysis for arsenic content in cutaneous lesions of 7 adult patients with arsenical keratosis and for Bowen's disease from 50 adult patients. Normal skin and a variety of cutaneous lesions and diseases from 119 patients were similarly analyzed. Ninety-five percent of the Bowen's and control patients had no previous history of arsenic intake. The data indicate that in lesions and in the adjacent skin, arsenic is present in increased amounts in a significantly greater proportion of Bowen's patients than in lesions of the control patients. The determinations from analysis for arsenic content in arsenical keratoses from 7 patients indicate that the proportion of patients with Bowen's disease and with arsenical keratosis do not differ significantly with respect to the presence of normal versus above normal arsenic values.

Our observations strongly suggest that arsenic could be one of the causes of Bowen's disease and that the internal and cutaneous cancer in these patients may well represent the systemic manifestations of this strong chemical carcinogen. Because of the similarities of Bowen's disease and arsenical keratosis and their associations with systemic cancer, we have wondered about arsenic as a common etiologic agent. The similarity of patients with Bowen's disease to patients with arsenical skin cancers as reported by Shu Yeh during this Conference, and the striking hetero-organic multiplicity of arsenic cancers as reported by Roth (*6*) support this concept.

ERYTHROPLASIA OF QUEYRAT

The present concept, as recorded by some observers (*8*, *9*), is that the premalignant lesion, erythroplasia of Queyrat of the glans penis, is Bowen's disease of that anatomical site. This interpretation is based on the striking microscopic resemblance of erythroplasia of Queyrat to Bowen's disease.

In 1959 we (*1*) reported data from 24 Caucasian male patients with penile lesions of erythroplasia of Queyrat. At that time we noted that the natural history suggested that erythroplasia of Queyrat was a different disease from Bowen's disease. This was based on the apparent lack of association of the penile disease with systemic cancer. This report presents our additional knowledge and observations of a larger group of patients with typical penile lesions.

Fifty male patients were studied. There were 43 Caucasians, 6 Negroes, and 1 Oriental. The age range at the time of first biopsy was 21 to 79 years; the median age was 52 years. The duration varied from 2 months to 25 years; the median duration was 2 years. Two patients (4%) had internal cancer and 3 others (6%) had cutaneous premalignant lesions of senile keratosis or basal-cell carcinoma. None of the 50 patients showed clinical or microscopic evidence of skin lesions resembling Bowen's disease or arsenical keratosis. The follow-up period for the 50 patients varied from 1 week to 13 years 8 months, a median of 5.2 years. Twenty patients were dead after a median period of 3.7 years and only 1 death was directly related to erythroplasia of Queyrat. Information regarding circumcision was available for 44 patients; 42 had not been circumcised prior to onset of the penile lesion.

Microscopically, erythroplasia of Queyrat of the penis shows the same atypical epidermal changes as Bowen's disease and arsenical keratosis. The main difference is involvement of the pilosebaceous follicle in the latter 2 lesions since this structure is lacking on the glans penis and foreskin. In 7 patients (14%) there was microscopic evidence of invasion of the underlying stroma, and 2 (28.5%) of the 7 had metastasis to the regional lymph nodes; 1 of these also had distant metastasis.

Chemical analysis of 6 lesions of erythroplasia of Queyrat for arsenic revealed significantly lower values when compared with the amount in lesions of Bowen's disease.

The difference in cancer proneness in 12 patients (24%), as compared to 75 patients with Bowen's disease (48.4%), is of striking statistical significance. This is particularly true in the relationship of the 2 diseases to internal cancer and other types of premalignant and malignant cutanneous cancer. Erythroplasia of Queyrat appears more prone to invade the underlying stroma as squamous-cell carcinoma (14%) as compared to invasion from lesions of Bowen's disease (5.2%). The natural history of our patients indicate that erythroplasia of Queyrat is a different disease from Bowen's disease. The prognosis is good in the former and less favorable for those with Bowen's disease.

SENILE KERATOSIS

The present concept (*10, 11*) in the medical literature is that senile keratosis is a premalignant skin lesion with recognizable clinical characteristics and is prone to occur on the exposed parts of the body. If untreated, approximately 20 to 25 percent of these lesions will eventually invade the underlying stroma as squamous-cell carcinoma, and in some instances there is metastasis to internal structures. The histopathological features are those of a carcinoma *in situ* and these may at times be confused with Bowen's disease.

In 1959 we (*1*) reported data from 35 patients with lesions of senile keratosis which had been removed prior to death or obtained at autopsy.

In this report we shall record our recent observations of patients with senile keratosis.

Anatomically, 216 lesions from 144 patients were studied histologically; these were single or multiple and occurred only on the exposed areas of the body. The majority of the patients were Caucasian males with an average age of 62 years at the time of biopsy or surgical removal of their lesions. The age range was from 26 to 86 years.

Microscopically, the typical features of senile keratosis are those of hyperkeratosis, parakeratosis—granular layer may be present and combinations of acanthosis and atrophy; loss of normal architecture of the deeper levels of the epidermis is observed. This loss of polarity is characterized by the presence of an atypical prickle-cell proliferation, pleomorphic cells, hyperchromatic nuclei, multinucleated forms, vacuolated cells, dyskeratosis, and abnormal mitotic figures. There may be elongation of the rete ridges which extend down into the underlying corium. Occasionally acantholysis and cleft formation within the epidermis are present, and malignant dyskeratotic cells may be seen in these spaces. The basement membrane at the dermoepidermal junction is intact. In the corium there is usually a chronic infiltrate of round cells, vascular ectasia, and senile elastosis. In 18 (12.5%) of 144 patients there were 26 (12%) lesions that showed histologic evidence of invasion of the corium, and these were diagnosed as senile keratosis with squamous-cell carcinoma. None of these lesions metastasized. After long-term follow-up of this latter group, and in our study of material from over 5,000 patients with lesions of senile keratosis, it is our conclusion that metastasis from senile keratosis with squamous-cell carcinoma rarely occurs. Examples of metastasis have not been seen in our material, but if it does occur it is probably much less than 0.1 percent. This is completely different from primary squamous-cell carcinoma (*de novo* type) which is an aggressive lesion, and from the evidence of our material (*1*) may metastasize in as high as 37 percent of the instances if not adequately treated.

Chemical analysis of lesions of senile keratosis from 17 patients revealed that 10 had arsenic values higher than normal in their lesions. This proportion was lower than in the patients with Bowen's disease and is significant at just under the 5 percent level of probability ($\chi^2 = 3.756$).

Preliminary data (*1*) suggest that there may be an association of senile keratosis with internal cancer and other premalignant and malignant cutaneous neoplasms. Even though senile keratosis and Bowen's disease have different clinical and histological features, they may act biologically in the same fashion. If the association of senile keratosis with internal cancer holds true after a thorough study of a large group of patients, this will be of significant importance and may well change the concept that this lesion is of concern only in regard to its cutaneous malignant potentialities. This will alter the present concepts regarding diagnosis, treatment, prognosis, and follow-up of this more common premalignant cutaneous lesion.

EXTRAMAMMARY PAGET'S DISEASE

In this paper we (*12*) shall review a clinicopathologic analysis of extramammary Paget's disease.

Forty patients with Paget's disease of the anogenital region were reviewed. The disease involved predominantly the older age groups and the median age at onset was 59 years. The average duration from onset to excision was 4 years. Most of the lesions occurred in the Caucasian race, with slight predominance in females. The only 2 Negro patients in the group were females. Anatomically, the lesions were located on the labium majus, perianal region, penis, scrotum, groin, pubic area, thigh, lower abdomen, perineum, and buttocks. The median diameter was 3.0 cm. Clinically, the lesions were erythematous to whitish-gray, elevated, ulcerated, crusty, scaly, eczematoid, and occasionally papillary. Over 50 percent of the patients complained of itching, and a few experienced pain or noted bleeding. The initial clinical diagnosis was often leukoplakia or Bowen's disease, and carcinoma. Surgical excision of the lesion was the treatment of choice. The process tended to recur or not to respond to X-ray therapy, radium, desiccation, and curettement. The more radical the surgical procedure the more favorable was the prognosis.

In 13 patients (34%) a cutaneous adnexal carcinoma (apocrine) was present immediately underlying the anogenital Paget's lesion; in 11 (85%) of the 13, regional or widespread metastasis to internal organs occurred. Seven patients (19%) had primary internal or extracutaneous cancer; 4 had adenocarcinoma of the rectum; 2 women had adenocarcinoma of the breast; and 1 woman had primary mucinous adenocarcinoma of the urethra. In 4 (57%) of the 7, regional or widespread metastasis occurred. Four patients had basal-cell carcinoma of the skin in addition to extramammary Paget's disease; of the 4 in this group, 1 had adenocarcinoma of the rectum. Statistical tests show an association between perianal extramammary Paget's disease and subjacent underlying glandular adnexal carcinoma, breast carcinoma, or regional internal cancer with or without metastasis. When compared with all other sites in the genital and pelvic region, this association is highly significant. The median follow-up period for 38 patients was 3 years. The median survival of 18 of the patients who died was 1.5 years after establishment of the diagnosis of anogenital Paget's disease. Information from 34 patients regarding malignant tumors in their immediate relatives did not reveal any with a history suggesting mammary or extramammary Paget's disease, but 5 families (14.7%) had internal cancer. Two had carcinoma of the stomach, 2 carcinoma of the colon, and 1 carcinoma of the pelvic area.

Initially the lesions were given a variety of pathologic diagnoses. The main microscopic changes of anogenital Paget's disease were hyperkeratosis, parakeratosis, acanthosis, and pale, vacuolated epithelial cells within the epidermis. The cells had clear or vacuolated cytoplasm, and the nuclei were either centrally located or compressed to the periphery. Cells with hyperchromatic nuclei, multiple nuclei, and mitotic figures were occasionally noted. The Paget cells appeared more numerous at the tips and sides of the rete ridges, probably because higher in the epidermis they tend to be compressed by the surrounding mature malpighian cells. Paget cells were often noted in the epithelial outer root sheath of the pilary complex and in the apocrine sweat ducts. Glandular and ductal arrangement of the cells, within the epidermis and, occasionally, extension into the corium as ductlike structures, may be noted, especially at the tips of the ridges and adjacent to the basement membrane of the pilosebaceous apparatus. The malpighian cells separating the Paget cells occasionally were compressed. A subepidermal inflammatory infiltrate of lymphocytes, plasma cells, histiocytes, polymorphonuclear leukocytes, eosinophils, and mast cells was usually present. Especially in those lesions with a dense subepidermal inflammatory infiltrate, there was prominent "dropping off" of melanin into the upper corium where it was both free and within melanophages. Capillary proliferation, vascular extasia, and fibrosis were usually seen in the upper and middle corium. Epithelial proliferation with Paget cells was noted in the ductal and glandular structures of sweat glands in lesions from 14 patients, but these changes were confined by an intact basement membrane and therefore were not regarded as adnexal carcinoma. Only those lesions with cells infiltrating the corium were interpreted as adnexal carcinoma. The carcinomas appeared to originate from apocrine structures, though origin in the eccrine sweat apparatus could not completely be ruled out. In the 7 patients with primary carcinoma of internal and extracutaneous organs, 4 presented a microscopic picture of adenocarcinoma of the rectum, 2 adenocarcinoma of the breast, and 1 mucinous adenocarcinoma of the urethra. Microscopically, in 15 patients with metastasis, the lesions appeared similar to the primary tumors.

Histochemically, the Paget cells contain cytoplasmic material which is positive with colloidal iron, alcian blue, aldehyde-fuchsin, and periodic acid-Schiff methods. The Paget cells contain predominantly an acidic mucopolysaccharide protein complex probably of the sialomucin type. Chemical analysis of 11 lesions of anogenital Paget's disease for arsenic revealed significantly lower values in comparison with the amount in lesions of Bowen's disease.

In summary, Paget's disease of the anogenital region is a distinct clinicopathologic entity. The disease involves epidermal apocrine structures as well as the pluripotential epidermis, pilosebaceous follicle and, in some instances, the apocrine dermal duct and glandular epithelium. Phylogenetically and histochemically, the apocrine structure is a mucin-secreting gland, and this feature, combined with its location in an area of entodermal origin overlapping with ectoderm, would account for the presence of mucin-secreting cells. An unknown carcinogenic stimulus may affect apocrine structures, epidermis, and glandular structures of the rectum and urethra. The latter 2 structures are of recognized entodermal origin. There is probably a multicentric effect rather than migration or metastasis of carcinoma cells into the epidermis. Cancer proneness is evident when it is considered that in addition to anogential Paget's disease, 23 (57.5%) of all patients had primary cancer in one or more locations. Twenty (52.6%) of 38 patients with follow-up had primary internal, extracutaneous, or adnexal cancer—18 of the anogenital area and 2 cancer of the breast. Of 18 deaths, 15 (83.3%) could be directly attributed to adnexal skin cancer or primary cancer of the rectum, urethra, or breast. Before or at the time of death, 17 (94.4%) of 18 patients showed evidence of cancer.

INTRAEPIDERMAL EPITHELIOMA OF JADASSOHN

The present concept of some observers (*13*) is that intraepidermal epithelioma of Jadassohn is a benign cutaneous keratosis, though Haber (*14*) reports the lesion can become a carcinoma *in situ* or an invasive carcinoma. At times its clinical and microscopic features may be confused with Bowen's disease. We shall report here our preliminary observations on a group of patients with intraepidermal epithelioma.

Histological study of 43 males and 33 females with single lesions revealed that these lesions were located on all parts of the body, except the palms, soles, and anogenital region; 41 percent were located on the lower extremities. The only Negro patient was a female. The age range at the time of first biopsy was 22 to 93 years; the median age, 50 years. The duration varied from 1 month to 24 years; the median duration was 4 years. The follow-up period for 72 patients varied from 1 to 30 years, a median of 5.8 years. Nine were dead after a median period of 4.8 years, and only 1 death was directly related to the intraepidermal epithelioma of Jadassohn. Three patients (4%) had internal cancer and 10 others (13%) had cutaneous premalignant and malignant lesions of senile keratosis, senile keratosis with squamous-cell carcinoma, malignant melanoma, or basal-cell carcinoma.

Microscopically, the typical features are hyperkeratosis, spotted parakeratosis, horn cysts, plaquelike acanthosis, and discrete nests of cells within the epidermis. These cells may appear basal or squamoid in type and are separated by prickles, but appear distinct from the immediately adjacent malpighian cells. These cells may be aggregated together in whorls and they contain melanin pigment. They may be uniform and cohesive or show evidence of anaplasia. In the upper corium, there is usually a mild round-cell infiltrate, and connective-tissue fibrosis. Six patients (8%) had microscopic evidence of local invasion and 1 of the 6 had regional metastasis. The latter also had carcinoma of the prostate. As long as the basement membrane at the dermoepidermal junction remains intact the lesion is relatively nonaggressive and usually remains so for many years.

Chemical analysis of 13 lesions of intraepidermal epithelioma of Jadassohn for arsenic revealed significantly lower values compared with the amount in lesions of Bowen's disease.

The difference in cancer proneness between 19 of these patients (25%) and 75 patients with Bowen's disease (48.4%) is of striking statistical significance, *i.e.*, patients with intraepidermal epithelioma of Jadassohn are less likely to have internal cancer or premalignant and malignant cutaneous lesions. The Jadassohn lesion is more prone to invade the underlying stroma as carcinoma when compared with Bowen's disease, but is probably less likely to metastasize. The prognosis is good in patients with intraepidermal epithelioma of Jadassohn compared to patients with Bowen's disease. No instance of both diseases occurring in the same patient has been found.

Intraepidermal epithelioma of Jadassohn is a distinct clinicopathologic lesion and, based on our observations, it should be classified along with the precancerous dermatoses.

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INFLUENCE OF AGING ON THE SKIN[1]

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SUMMARY

Conditions in man are described except when otherwise stated. The influence of age is best exemplified by the special ages of maximum prevalence characteristic of different cancers. With the long human lifespan there are many varieties of cancer with long latent periods. Actual latent periods are often shorter than those calculated. Ideas about what happens during latency are discussed and some chemical data are presented. Some older persons and all older cells are more resistant than young ones to most carcinogens. The age factor in susceptibility of a strain of mice to a carcinogen is overruled by a genetic factor. For this there is no precedent in man.—NCI Monogr. No. 10: 335–348, 1963.

I SHALL attempt to describe briefly the influence of aging on the biology of cutaneous cancer. My remarks will relate to man except when specific reference is made to animals.

INFLUENCE OF AGE ON MAXIMUM PREVALENCE OF CANCER

The terms prevalence (frequency), incidence, etc., will be used as by the U. S. Public Health Service in its survey of cancer illness, but most of the rates available to me do not relate to 100,000 age-adjusted populations.

That malignant tumors (cancers) make their appearance primarily in certain age brackets is the most outstanding single manifestation of the influences of aging.

Congenital cancers present at birth were first and most carefully evaluated by Wells (*1*). He observed that they are almost all different from those occurring in adult life except for the sarcomas. That they develop with unusual speed is evident, owing to the limitation in time of intrauterine life and the considerable delays within this period experienced by most of them in getting started. It is beside the point that a few fetal cancers develop from cancer cells and chemical carcinogens that invade the fetus from the mother.

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This rapid development of congential cancers is to be expected because the younger an organism, a tissue, or a cell is, the greater seems to be its susceptibility to malignant transformation.

After birth the ages of maximum frequency of many cancers have been determined. Mulligan (*2*) cites 52. All cancers seem to have ages of maximum frequency. The major ones are supplied by Hueper (*3*), quoting Hortz.

	Years
Testicles, kidney, and adrenal	0–4
Bone	15–25
Testicles	30–35
Brain	40–45
Female sex organs	50–55
Skin and abdominal organs	60–65
Prostate	70 plus

Note the 2 ages of maximum incidence of cancer of the testicles. Oliver (*4*) described 2 ages of maximum frequency of breast cancer and it is likely that still others will be reported. This incidence must be interpreted as occasioned by age changes in susceptibility and in exposure to carcinogens.

INFLUENCE OF LIFESPAN ON CANCER

In the words of Dublin (*5*), "The life span properly conceived is the limit beyond which human life does not extend even in the most favorable circumstances." He places this at 100 years and says that there is no reason to believe that it "has varied appreciably within historic times."

The ages of maximum incidence of cancers within the short lifespans of animals cannot be accurately superimposed on those already given for man within his much longer lifespan.

Speaking about the production of tumors in mice through applications of methylcholanthrene, Strong (*6*) says: "The variety of tumors can be increased by hybridization, by the method of application, the dose and vehicle used, et cetera."

It is a moot question whether *Homo sapiens*, since it evolved, has suffered more hybridization than *Mus musculus*. Probably *H. sapiens* has endured as a species longer than *M. musculus* and has enjoyed a longer time in which to produce hybrids, but it exists in fewer varieties than *M. musculus*, capable of producing hybrids by being cross-bred. Moreover, because of its much longer lifespan *H. sapiens* is unable to produce as many hybrids by crossbreeding of varieties in the same unit of time as *M. musculus*.

Although the variety of susceptible cells in man may not greatly exceed that of mice, the number of each kind certainly does, since we are much larger. It is agreed that each cancer is a law unto itself and that no two are really identical. Consequently, equal exposure to a particular carcinogen of a larger number of susceptible cells in man would be expected to result in a larger variety of cancers than in mice having fewer susceptible cells.

Since we live in so many more different environments than mice, we are probably exposed to a wider range of carcinogens. For this reason, also, the larger variety of human cancers is expected.

Between adequate exposure to carcinogens and the recognition of resultant cancers there is always a period of latency, distinctly longer in man than in mice. This should be discussed in the framework of some knowledge of the different kinds of latency.

KINDS OF LATENCY

With the latency of rest we are not now concerned except to describe it briefly in connection with the latency of activity in cancer development. The two are probably in some way related.

The latency of rest is sometimes observed in both neoplastic and normal cells. In the former, it is exemplified by recurrences after almost, but not quite, complete removal of primary cancers. The previously malignant cells apparently remain asleep for long periods and manifest none of their destructive properties.

Stewart (7) thinks "the all-time record" is that of an adenoid cystic carcinoma of the salivary gland, first observed in a 15-year-old patient. The carcinoma was removed when the patient was 29 years old and he died at the age of 80. Stewart considers this to be a period of delay of 65 years unless, in the interim, a second primary tumor developed in the same position from some normal cells of the salivary gland not removed at the operation. This is a real possibility.

During the period between its first observation at the patient's age of 15 and removal of the tumor at age 29, the tumor must have progressed somewhat and could not have been latent while still progressing. Moreover, since in an unmeasured period before it killed the patient it was certainly not latent, we cannot, with assurance, sharply estimate the actual length of latency in this case or any other like it. Yet such observations of delayed recurrence are so frequent that periods of fairly long latency must often occur, and during them the cells must be more or less at rest.

This latency of rest is also seen in many non-neoplastic cells. Ectopic, yet normal looking cells are found in small clumps far out of place in the digestive tract. Examples are gastric epithelial cells in the upper end of the esophagus and intestinal epithelial cells in the gastric mucosa. These ectopic cells, found by chance, differ from their neighbors in showing few if any signs of activity. They may have been quiescent for a long time. It is claimed that these kinds of cells on occasion undergo malignant transformations.

Nevi are congenital lesions that may persist for a lifetime without increase in size or noticeable change. They merit the designation "latent." Marginal ones occasionally become malignant.

Latency of activity is different. For us this consists of rapid production or maturation of carcinoma cells. It is still generally conceded that, within the period of latency after exposure to carcinogen, two steps are being taken toward outright malignancy: initiation and promotion. Latency in sarcomas has been less studied.

MEASUREMENT OF LATENCY

Disconcerting differences prevail in the results of measurement of latent periods in different groups of cancers. In occupational cancers, the delay since the last hazardous exposure is placed at 10 to 20 years. These cancers result from accumulations of carcinogenic insults beyond the limit of tolerance as in heavy cigarette smoking, long and repeated exposures of the skin to strong sunlight, and oft-repeated exposure to radiation.

If, on the other hand, one measures the latent periods from accurately timed starting points, as in the cases of single trauma carcinomas from Leighton and Schmidtke's (*8*) Barnard Hospital series, the latent periods are found to be much shorter than in occupational cancers, with an average of about 20 months. The shortest reported delays noted in two squamous-cell carcinomas of the back of the hand and face, respectively, were only 5 weeks. Actually the delays were even shorter than recorded, because it always takes some time for cancers to develop to the point of visibility and recognition. This should give us pause if we feel inclined to say that a small lesion appears too promptly to be malignant or that it is so tiny that it can be ignored.

All 42 cases involved trauma of the skin and all resulted in carcinomas. There were no cancers of the breast or bone sarcomas. Our conclusions about latency in cancer production are based almost entirely on skin cancers that are readily visible. We have fair estimations concerning the range of latency of lung cancers for which the responsible carcinogens are known and the duration of exposure dated. But for carcinomas of the digestive tract and its appendages, constituting a majority of all fatal carcinomas, the responsible carcinogens are usually unknown and recognition of the resulting cancer is often delayed. Both the inception and the conclusion of the latent period generally are in doubt.

With appreciation of these difficulties we conclude that the latent period of epidermal cancers *per se* bears approximately the same relation to the lifespan in man and the few other mammals investigated. But our estimates of the latent periods are apt to be unduly long in humans. I have the impression that exposure to carcinogens in man is often subminimal and is evidenced by the fact that cancers of particular kinds develop only in a small fraction of those exposed, whereas in experiments on animals the exposure is usually sufficient to produce cancers in almost all of them.

WHAT HAPPENS DURING LATENCY?

The major project of the Barnard Free Skin and Cancer Hospital during more than 10 years was to find out chemically with suitable microscopic controls what happens during latency. We fortunately discovered a method by which epidermis can be cleanly separated from dermis for chemical analysis and consistently used it in the collection of material suitable for analysis. Time permits only mention of the changed chemical composition of transplantable methylcholanthrene-induced squamous-cell carcinomas in mice compared with that of normal, untreated epidermis.

No change was observed in some important cellular constituents while others increased and decreased in amount as indicated below:

	Properties of squamous-cell cancer compared with those of normal epidermis in mice.
Increase: (50% or more) ↑	Lipide phosphorus: dry-weight ratio, succinic dehydrogenase activity, adenylpyrophosphatase activity, arginase activity, lysine, tryptophan, isoleucine.
Increase: (less than 50%) ↑	Water content, choline, leucine, methionine, valine, phenylalanine, threonine.
No change:	Ascorbic acid: nucleoprotein phosphorus (NP) ratio, specific activity of P^{32} in phospholipide fraction, inositol (wet-weight basis), histidine, glutamic acid, cystine, arginine.
↓ Decrease: (less than 50%)	Potassium: NP ratio, sodium: NP ratio, magnesium: NP ratio, cytochrome oxidase, deoxyribonucleic acid (wet-weight basis), cytochrome *c*, *p*-aminobenzoic acid (wet-weight basis), vitamin B_6 complex (wet-weight basis).
↓ Decrease: (50% or more)	Calcium: NP ratio, iron: NP ratio, copper: NP ratio, zinc: NP ratio, nonprotein nitrogen (wet-weight basis), urea (wet-weight basis), ammonia (wet-weight basis) total free amino acids (wet-weight basis; chromatography), biotin (wet-weight basis), Ca^{45} uptake and retention, free calcium.

Gluckmann proposed a method of classifying the types, and to some extent the ages, of the different kinds of epidermal cells involved, which would enable us to calculate how direct was the bearing of chemical analyses on those cells becoming malignant. Davibhadhana, cooperating with us, investigated the numerical proportions of different cells in our standardized carcinogenic series. Still no feasible way of checking out results of analyses of living and dead cells and intercellular materials was developed, which would leave only those bearing on direct cellular carcinogenesis.

It is possible, even likely, that our analyses missed some stages of carcinogenesis that took place in hair follicles because these were not included with epidermis when it was removed from dermis for analysis. Moreover, only in our later studies was the importance of the hair follicle cycle recognized by us [Liang and Cowdry (*9*)], so that, in our earlier work, treatment with carcinogen was not started with mice in the resting phase. Argyris (*10*) points out that many more skin tumors develop in the resting phase after a single application of 7,12-dimethylbenz[*a*]-anthracene (DMBA) in benzene than when the follicles are growing. This is not what one would anticipate if the increased mitotic rate during growth increases the susceptibility to chemical carcinogens.

Bock's (*11*) observations tend to confirm those of Argyris insofar that he found slightly more hydrocarbon in skin in which the hair was "resting" than in skin with growing hair, and that metabolism or transport of carcinogen from skin with growing hair was somewhat more rapid than from skin with "resting" hair.

The results, however, do justify some important conclusions. One is that the chemical composition, thrown out of joint in so many ways, sets the stage for the occurrence of survival mutations, some of which may have been malignant. But they are not evidence that one or more mutations actually took place. Moreover, they have been neither confirmed nor denied by other workers. Our group, numbering at one time or another over 50, who wrote their own papers and to whom credit is given in my book (*12*), seems to comprise the only investigators of chemical changes during epidermal carcinogenesis.

It is generally conceded that experimental epidermal carcinogenesis is divisible into two parts: a brief stage of initiation during which a malignant mutation takes place and a longer one in which the young cancer cells thereby created, or their descendents, are promoted to invasive cancer cells.

Our chemical findings do not militate for or against the Millers' (*13*) enzyme deletion hypothesis or Berenblum's gene liberation hypothesis. Berenblum (*14*) wrote that his hypothesis does not postulate the action of either a mutagen or a virus, but it would seem that the liberation of a gene, or genes, would itself constitute a mutation in the genetic equipment of the cell.

Foulds' (*15*) independent progression and incipient neoplasia hypothesis does not appeal to me, because, in the extensive areas of incipient neoplasia reported, cells widely distributed must somehow have undergone a sudden change in the same direction, which could hardly be mutational, for only a microscopic percentage of cells affected by a carcinogen is thought to undergo mutations. True, all these cells exhibiting incipient neoplasia could have resulted from virus action, and their transformation into mature cancer cells capable of invasion could likewise have resulted from virus action, perhaps even from action by one and the same virus.

Iversen and Evensen (*16*) are approaching, with refreshingly new techniques, the study of skin in hairless mice. First, they point out several established facts:

A cell has to double its content of deoxyribonucleic acid (DNA) before division, and after this, each of the daughter cells contains the same amount of DNA as the mother cell did originally.

DNA synthesis takes place during the interphase and is completed some time before mitosis. This period is called the DNA synthesizing time.

Part of thymidine injected into an animal will be taken in exclusively by cells engaged in DNA synthesis as long as thymidine is available in the circulation.

With thymidine labeled with tritium H^3, cells synthesizing DNA can be registered autoradiographically.

The radioactivity of a cell nucleus, labeled with tritiated thymidine, is reduced by one half at each mitosis.

In their experiments they calculated with the aid of Colcemide, which arrests mitoses in the metaphase, the mitotic duration, measured from the dissolving of the nuclear membrane to the end of the telophase, to be 1.13 hours. They then investigated epidermal cell proliferation for 16 days after a single application of 0.005 ml of a combined solution of 1 percent 3-methylcholanthrene and 1 percent phenanthrene in benzene. They concluded: 1) that the initial raise in mitotic count and the initial decrease in the number of labeled cells are occasioned by a block in mitosis and inhibition of DNA synthesis; 2) that the low number of labeled cells observed after painting is to be interpreted as a shortening of the mitotic duration and of the DNA synthesizing time, the rate of cell renewal being increased; 3) that the subsequent increase in mitotic count, and in number of labeled cells, is mainly brought about by prolonged mitotic duration and an increased synthesizing time.

While Iversen and Evensen have devised and used a much better technique than hitherto employed, which has for the first time supplied statistically valid data on changes in mitotic duration and DNA synthesizing time, their observations were made only on 4 sections 5 to 7 μ thick through the painted areas that must have only partly included many cells both in interphase and mitosis. Consequently the mitotic ratios were not based on as many as the 15,000 nuclei examined in wholemounts of epidermis at each time interval by the Barnard group. In other words, their survey was sharply limited. Clearly, Iversen and Evensen's investigations should be extended to the development of malignant cells.

These concepts and observations are based primarily on epidermal carcinogenesis. They are not intended to explain the genesis of similar squamous-cell carcinomas from other epithelia, of which the body possesses a considerable variety. They do not explain the development of sarcomas from cells of mesenchymatous origin.

It is advisable to remember the ideas expressed by Orr (*17*) on the permutation of the stromal tissues during carcinogenesis. We shall hear from him later, but first let me say that I am impressed by the great length of the latent period in human epidermal carcinogenesis, sometimes extending to 40 years or more. If cancer cells are created by a mutation at the beginning of the period of initiation, and thereafter remain at rest until promotion is consummated, we wonder what happens to them in the long years during which other epidermal cells rise to the surface, age, die, and are desquamated. We ask ourselves whether resting, but potentially malignant, cells can hold back and save themselves from a similar fate.

Orr's (*18*) keynote sentence is in his abstract for this conference: "I remain convinced that the mechanism of epidermal carcinogenesis cannot be explained in terms of a direct carcinogen-epidermal cell reaction." He then cites experimental data:

"Billingham, Orr, and Woodhouse showed that if pure epidermal grafts were transferred from a body-site, which had received a fully adequate carcinogenic treatment, to another site, tumours never developed from the transplanted epithelium; more unexpected was the finding that the same applied to *thin* Thiersch's grafts. Even when the grafts are subsequently treated with the co-carcinogen croton oil (Marchant and Orr) they do not give rise to tumours. On the other hand, the original carcinogen-treated site, whether re-epithelialized by natural regeneration or by grafting with untreated epidermis, develops tumours at approximately the expected rate. Reasons have been given for regarding it as unlikely that the proximal parts of the treated hair follicles are responsible for this."

Evidently the dermis does somehow participate in epidermal carcinogenesis. Evidently, also, it is unsafe to assume that similar events take place in the latent periods of all malignant tumors which develop in very diverse tissue-fluid environments.

INFLUENCE OF AGE OF INDIVIDUALS ON BEHAVIOR OF CANCER

According to Aub and Lombard (*19*), from the clinical point of view, carcinomas (not specifying which) often appear to run a milder course in old than in young people, though these neoplasms in old people appear not to be distinguishable from neoplasms of the same sort in younger people. How much this is due to differences in the cancers themselves and in their environments within our bodies is not clear.

To specify in just what respects the environments are different and to relate these to the behavioral differences are quite beyond us. Logically, environmental factors require consideration not only between the old and the young, but as operating on cancers through the lifespan. They are most manifest for each kind of cancer sometime before its maximum incidence while it is most subject to influence by them.

Aub and Lombard have also made observations on cancer survival rates in relation to ages of patients. First, they presented data on 1,683 breast cancer patients, which they considered in three groups:

Localized with microscopic diagnosis	578
With metastasis, with microscopic diagnosis	871
Clinical diagnosis only	383

The percent survival rates showed "by and large, that the group over 70 years of age have as good or better survival rates than those under 70." Evidently most old women with breast cancer are not so handicapped by their disease.

They then determined the survival rates for all of 14,487 individuals with cancer and found that "at the end of the first, second, third, fourth and fifth years the 70–79 and the 80–89 groups have greater survival rates than the younger age groups."

Obviously these calculations smooth out any individual differences that may exist for particular sorts of cancer. It could be that for some cancers the survival rate is greater for older people and for others less than for young people. But the general trend is certainly toward higher survival rates for these old persons than for younger ones. This supports their previously stated idea that cancers run a milder course in old people.

That the cancers in old people are somehow held down to a milder course also corresponds well with the evidence from other sources that transplanted tumors do not grow as well in old animals as they do in young ones.

INFLUENCE OF AGE ON SUSCEPTIBILITY TO CANCER

Since cancer is *par excellence* a cellular problem, it is customary to dilate on cellular specificity *per se*. This is artificial and narrow minded. Actually the influence of aging on cancer susceptibility depends not only upon the cells as vital units, but also on the whole body as a vital unit in which the cells live and themselves age. It is proper, therefore, first to consider the influence of aging cells on their susceptibility, and second, that of aging of the body on access of carcinogens to susceptible cells.

As is customary, the older and more differentiated a cell is, the more resistant it is to carcinogens and the less it is able to undergo mitosis. Aging tends to withdraw the malignant potency of cells more rapidly in some types than in others. This is not unrelated to the observation already made that cancers in old people are inclined to be more sluggish and less aggressive than in young ones.

The reverse also holds, for in general the younger and less differentiated a cell is, the more susceptible it is to carcinogens and the most undifferentiated tumor is the most malignant.

To reduce to a common denominator the carcinogenic "kick" received by susceptible cells is impossible. Evidence is convincing that, when a second carcinogen acts, it may inhibit or enhance the activity of the first and that some tumors require for their initiation not only carcinogens but also certain endocrine products as well as genetic susceptibility. Moreover, at the human level, our tissues are more frequently afflicted by a greater multiplicity of weak carcinogens than by single strong ones. We must also be on the lookout for promoting and inhibiting factors.

It is generally thought that young tissues and undifferentiated tumors, both with high frequencies of mitosis, are the most susceptible to carcinogens, and, when the tumors are most malignant, disappear most extensively by radiological treatment when this is first instituted. Radiosensitivity diminishes and resistance to subsequent treatments sets in. Thus X ray is both mildly carcinogenic and therapeutic.

Attempts to discover whether in a given cell type its interphase or mitotic phase is the most susceptible to carcinogens have not yielded precise results. In response to my inquiry, Leonell C. Strong has kindly expressed the following opinion: "As a general rule it is concluded that the late prophase is the most effective stage for the mutagenic action of X rays. However, in the case of chemical mutagens providing the chemical can get within the cell the most effective stage may be the interphase, or resting stage. Of course the mutations may not appear for three or four generations."

With chemical mutagens, including viruses, the permeability of the plasma membrane, or the ability of the cells to take them in by pinocytosis, may be influential. Perhaps the normal resistance to the entry of some of them may be slightly enhanced if and when the number of layers of molecules in the plasma membrane are increased, owing to increased surface tension. When cells decrease their surface area by rounding up into spheres preliminary to mitosis, permeability would be expected to decrease temporarily but not necessarily noticeably. While all this is highly speculative, it would nonetheless seem to indicate that the interphase may be more susceptible to chemical carcinogens because of its greater surface area.

But it would be shortsighted to get the idea that all carcinogens must enter susceptible cells to bring about a mutation, for there are reasons to suppose that influences outside susceptible cells, such as anoxia, can be carcinogenic.

One is tempted to entertain the hypothesis that cells of several varieties, when dedifferentiated during mitoses, are reduced to about the same level of susceptibility to some carcinogens, perhaps to most.

This highlights the importance of attempts to evaluate the influence of aging on the accessibility of susceptible cells to chemical (and viral) carcinogens. Nothing need be said about X rays and other penetrating physical carcinogens. The penetration into epidermis of carcinogenic ultraviolet radiation is but slight. The much thicker corneal stratum of human epidermis wards off much more of it than the thin stratum corneum of mice.

Accessibility of susceptible cells to external chemical carcinogens depends on entry through the skin, digestive and respiratory tracts, and their passage via the blood stream to the tissue-fluid environments of susceptible cells. How this accessibility is modified by advancing old age is a long story.

Percutaneous absorption has been fully described by Rothman (*20*), but adequate data are lacking as to its degree of modification in old age and the influence of the modifications on the entry of substances capable of causing cutaneous cancers.

According to measurements of thickness of epidermis in the antecubital space made by Evans, Cowdry, and Nielson (*21*), there is little difference between young and old persons, but in more exposed areas of skin, epidermal atrophy may be noticeable. In wholemounts of epidermis separated from dermis, Cowdry, Cooper, and Smith (*22*) noticed a general smoothing out of epidermal rete pegs and a thinning of epidermis. Actually it must be admitted that the epidermis and its derivatives exhibit with advancing age an increasing lack of stability. In small and even large areas hypoplasia (atrophy) vies with hyperplasia, depigmentation with pigmentation, and demineralization with hypermineralization. This can hardly be without influence on percutaneous absorption of substances, some of which may be carcinogenic.

Although the digestive tract and its appendages constitute a diverse and fertile field for absorption of potential carcinogens, the etiology of the many cancers that develop therein is almost entirely unknown. Here also age differences are to be expected.

It is the respiratory system that illustrates best the letdown in efficiency of the mucociliary clearance mechanism with advancing age and almost coincident with increase in bronchogenic carcinomas.

Endogenous carcinogens do not have to pass these defenses. Both must survive metabolic breakdown, or detoxification, in the blood stream and other body fluids before they impinge on susceptible cells. Their fate can be predicted to some extent by knowledge of their chemical composition.

Lastly, the tissue-fluid environments of all susceptible cells are far from uniform, in my concept of heterostasis. Probable alterations with age in these, in which all chemical carcinogens influence the susceptible cells, though but little known, must not be ignored.

INFLUENCE OF MATERNAL AGE ON VIRUS-INDUCED TUMORS

Some interesting observations of this have been made on mice. In four different strains Dmochowski (*23*) discovered that ". . . the increasing age of the mother leads to a decrease in the mammary tumor incidence in her descendants, irrespective of the number of litters born to the mother and of the number of litters born to the descendants."

He points out: "Advanced parental age was observed to decrease the incidence of spontaneous mammary tumors and of hepatomas in another strain of mice (Mühlbock (*24*)) though in a somewhat different manner."

But he also calls attention to Strong's (*25*) observation to the contrary, that the malignant character of chemically induced tumors increases with increasing age of the mother; this is likewise true in mice. This is a surprising discovery difficult to explain, for, in spontaneous human cancers, all of which are somehow induced, the malignancy in the mother herself decreases with age. There is no evidence whether at the human level the malignancy of the cancers in the offspring increases or decreases with maternal age.

RELATION OF GENETICS AND AGE SUSCEPTIBILITY TO CANCER

Obviously both genetics and age are lifelong influences. Examples of genetic susceptibility to cancer are many and I shall not attempt to catalogue them.

Taylor (*26*) gave a classical description of a family in which there were many cases of the dry, pigmented skin lesions of xeroderma pigmentosum. This affliction is certainly inherited, but vertical inheritance is impossible because children with it die before they can become parents. The skin is extremely sensitive to sunlight, and in this country the disease is invariably fatal because of the squamous-cell carcinomas that develop.

The remarkable Caribe Cuna Moon-children, as described by Keeler (*27*) and at this Conference, are albinos. Indeed in the Caribe Cuna Indians there appears to be more albinism than anywhere else in the world. These albino Moon-children have skin like children with xeroderma pigmentosum elsewhere which is unduly sensitive to sunlight so that they frequently develop xeroderma pigmentosum, followed by skin cancer. Some grow to maturity and have offspring. But these are few and it is impossible to trace them back through several generations because of the absence of records. Since the maximum incidence of this variety of skin cancer is 60 to 65 years of age, the responsible genes for xeroderma pigmentosum functioned about 50 years earlier than usually dictated by age susceptibilities.

Cowdry and Suntzeff (*28*) compared carcinogenesis in young and old New Buffalo mice. They found that these squamous-cell carcinomas, resulting from application of methylcholanthrene, appeared in larger numbers at all ages studied in young and old mice, despite the fact that the applications to both groups were as nearly as possible identical.

A curious thing happened in a similar experiment with young and old mice of strain CBA. In these the cancer response was nearly the same in both young and old mice, for the cancers did not appear earlier and in lesser number in the CBA strain. Since the only apparent difference between the two strains was genetic, it would appear that a gene or genes in the CBA mice suppressed the influence of age in increasing youthful susceptibility which was so clearly suppressed by the New Buffalo mice.

The genetic factor in susceptibility seemingly overruled the age factor. But the age factor is genetic. Consequently, we have in xeroderma pigmentosum and in this carcinogenic series influences that overpower the age factor in production of the same kind of cutaneous cancer.

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ALBINISM, XERODERMA PIGMENTOSUM, AND SKIN CANCER[1, 2]

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SUMMARY

Albinism and xeroderma pigmentosum are similar in that both groups of patients have dry skins, irritated eyes, large freckles, keratotic spots, and skin ulcerations brought about as a response to light. Both conditions are inherited as simple, recessive entities. Under certain conditions, both develop skin cancers. They differ in that hair and eye pigment in xeroderma is normal, but greatly deficient in albinos, resulting in nystagmus. Xeroderma has a lower threshold of sensitivity than has albinism, often developing many malignancies by 6 years of age in the temperate zone, whereas Caucasian albinos in the temperate zone seldom develop cancers. Cuna albino Indians in the tropics, however, often form skin cancers in their late teens or early twenties and all show actinic keratoses at the age of 3. Xeroderma is quite rare, possibly having an incidence on the order of 1 in 20,000; albinism occurs about once in 10,000. Among the Navajos it appears twice in 10,000 and, Cunas have 45 albinos in 10,000. The gene for xeroderma pigmentosum may be partially sex-linked, whereas, that for albinism is autosomal. The malignancies of xeroderma are usually basal-cell and squamous-cell carcinomas; melanocarcinoma also occurs; albino skin cancers among the Cunas are almost always squamous-cell carcinomas. The heterozygotes of xeroderma pigmentosum may be freckled; those of the albino are not freckled but may be intermediate in skin color between albinism and normal pigmentation in both Caucasians and Amerindians. Cuna albinos (Moon-children) present an extensive syndrome of pleiotropic effects that can be recognized in nystagmus, decreased body length and weight, a weak hand squeeze, lack of sweating and low specific gravity, fine head hair, growth of hair on appendages, lack of energy, deficient male sex hormones, and stubborn temperament, in addition to their propensity to develop skin cancers.—NCI Monogr. No. 10: 349–359, 1963.

ALBINISM and xeroderma pigmentosum have been linked together for discussion on this program because they have a number of clinical signs in common: light-sensitive, dry skin, photophobia, freckles, keratotic

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patches, and a tendency to form skin cancers. However, these two entities differ in many respects, as we shall note here.

XERODERMA PIGMENTOSUM

This condition is described as a precancerous disease of the skin usually first manifested in childhood. It is characterized by large freckles, atrophic areas, and keratotic spots (fig. 1).

The first signs of xeroderma pigmentosum in childhood may be a crowded distribution of enormous freckles on all exposed areas, dry skin with dandruff, and, especially, irritation of the conjunctiva. Soon keratoses, warty excrescences, and ulcerations appear. These signs are quickly followed by the profuse development of malignancies. Hair and eye pigmentation are normal in this disease, and there are no congenitally associated eye defects. Because irritation of the eyes produces the initial discomfort, the xeroderma pigmentosum patient may be brought first to the Ophthalmological Clinic where the preclinical signs of cancer are not likely to be recognized.

Histology

Atrophy, acanthosis, and hyperkeratosis are seen in histological preparations together with deposits of melanin in the basal layer, the lowest layers of the stratum spinosum, and chromatophores of the upper epidermis. Areas of the epidermis may resemble lentigo malignum or junctional nevus. Basal-cell, squamous-cell, or melanocarcinoma may complicate the picture of xeroderma pigmentosum (fig. 3).

There is basophilic degeneration of the collagen with atrophy in the dermis. The elastic fibers are also degenerated. Neoplastic elements are usually basal-cell or squamous-cell carcinomas, although spindle-cell carcinomas do occur.

Treatment

The usual treatment for xeroderma pigmentosum is electrocautery or surgical removal of the malignancies as they appear. I have known a case in which a hospital staff did not recognize this rare syndrome and carried out a series of intensive X-ray treatments that probably aggravated the condition, although I have found radiation recommended in a pathology text.

Prognosis

At present, there is no cure for xeroderma pigmentosum, and the skin malignancies increase rapidly in number, in spite of treatment, until metastases finally spread to the internal organs and cause death.

Heredity

Xeroderma pigmentosum is familial, that is, a recessive disease. Being quite rare, it occurs due to consanguinity much more often that to random mating. Both Fisher (*1*) and Haldane (*2*) agree that there is mathematically sound evidence for assigning the recessive casual gene for this disease to the homologous portion of the X and Y chromosomes, because of partial sex linkage. However, their maps do not agree in the finer details of linear order of genes or the number of units that separate them. The partially sex-linked associates include the genes for achromatopsia and congenital night blindness. On the basis of this work, chromosome maps, for the homologous portions of the X and Y chromosomes of man, have appeared in textbooks for more than a decade and locate the zeroderma gene and others in this region. Recently, however, Morton (*3*) has criticized this mathematical treatment, declaring that the results obtained are merely the effect of a cryptic statistical error created by an improper selection of data.

Heterozygotes for xeroderma pigmentosum frequently exhibit freckled, sensitive skins, but do not develop the disease. The condition probably occurs less than once in 20,000.

Figure 1 shows a case of xeroderma pigmentosum that I first recognized at the Massachusetts General Hospital in its preclinical stages when dry skin, freckles, and dandruff were the only signs of the disease. Conjunctival irritation had begun, but was so slight that it might have been caused by rubbing the eyes while crying or by a slight allergy. A year and a half later, at the time of the photograph, five cancers had been removed from the cornea, cheek, and back of the neck.

ALBINISM

In contrast to xeroderma pigmentosum, albinism is recognizable by snow-white hair and a great reduction in ocular pigmentation producing the nystagmus so characteristic of this disease. The exposed, light-sensitive skin takes on a characteristic pink color, softened by its white lanugo. There is some suggestion of reduced metabolism. In the temperate zone, and with no undue exposure to sunlight, these patients do very well. Because of their ocular difficulties, albinos are frequently found in schools for the blind.

Treatment

In the temperate zone there is no treatment recommended for the disease aside from protective glasses, protective clothing, and staying out of the sunshine.

Prognosis

The prognosis for albinism in the temperate zone is excellent, and it is seldom thought of as a disease but rather as the sensitive skin accompaning an eye defect. Skin cancer rarely occurs in these albinos, if the precautions mentioned are taken.

Heredity

True albinism in man, as in all mammals, is a simple recessive, mendelian characteristic dependent on the presence of a single pair of genes. In forms such as the mouse, rat, rabbit, and cat, these genes are members of a series of multiple alleles or alternative forms of the same gene molecule. Other members of the series include chinchilla and himalayan. There is some evidence that at least one other member of the series may exist in man, characterized by pink eyes and some pigmentation in the hair, but the evidence is not conclusive.

The frequency in Europeans is estimated at about 1 per 10,000. In blonds of the Caucasian race, heterozygous for albinism, the eyes may be sensitive and the hair color lighter than that of true blonds in the family not carrying the albino gene. In like fashion, mice greatly diluted in coat pigmentation will often be even lighter in fur color when they carry the albino gene heterozygously.

Thus far, we have described the conditions for Caucasians having albinism in the temperate zone. However, for Caucasians and even for darker races in the tropics, albinism may have a very severe, cancerous import due to the actinic rays of the blazing sun.

Caribe Cuna Moon-Child Albinos

For 13 years I have been in touch with the primitive Caribe Cuna Indians of San Blas, who live mainly on the string of offshore atolls that skirt the Caribbean coast of Lower Panama. I have visited them 8 times. Here we find the highest incidence of albinism in the world, now numbering about 45 per 10,000. Less than 95 individual albinos, called Moon-children by the natives, are available for study today. I say "less than 95" because the number I first counted seems to be decreasing—partly due to infanticide of the newborn.

Among the Cunas, albinism shows several characteristics not noted generally in Caucasian albinos of the temperate zone (fig. 2). Fine, white hair, sometimes an inch and a half long, grows on the lower arms and limbs of Cuna albinos, whereas the skin of the brown Indian is completely devoid of body hair. Head hair is finer in diameter than normal hair. Short, fine, white hair frequently grows on the sides of the face and even on the rim of the ears. Hard, keratotic spots are mingled among large freckles, and the skin presents a dry, senile appearance. It becomes thickened with wrinkles on the back of the neck at a very early age and is greatly thickened on the backs of adult males. Cuna albinos do not sweat. They are slightly shorter and lighter in weight than are brown-skinned Indians (*4*).

San Blas albinos lack energy and cease to be active in athletics by 13 or 14 years of age, taking over the job of referee or manager of the team. When children, they constantly carry infections of the nose and throat and appear to be deficient in the building of antibodies. On the skin of Cuna albinos mosquito bites become infected. The albinos seem to lack energy and have a low metabolic level. The power of the hand squeeze is reduced. The male sex drive is low in albinos and they seldom marry. The voice is high-pitched in males as though deficient in male sex hormones, and ultraviolet spectrum analysis of the urine reveals almost complete absence of the steroid estrogen peak in several teen-aged albino boys examined. However, Cuna Moon-child females are fertile although they mature late (*5*).

The weakness of albinos is rather general among mammals (*6*). Because of this weakness Cuna Moon-child albinos occupy a peculiar position in tribal society (*7*). The town council calls upon them for less rigorous physical labor than exacted from brown-skinned Indians.

It requires a certain amount of basal metabolism to commit crimes. Sex offenses are highly taboo, and until recently the offending parties were killed as well as the resultant bastard child. Albinos are said to sin less, in these respects, than other persons and, therefore, to be on better terms with Tiolele the Sun god. Because of this better behavior on earth, Moon-children will receive better houses in heaven, and others will not be permitted even to look into their heavenly mansions unless they have made friends with 8 Moon-children on earth. At the time of eclipse only the albinos are holy enough to rush out with bows and arrows to shoot and kill Achusimmutupalit, the Earthmother's Placental Monster, who is trying to end the world by swallowing up the sun or moon (*8*).

On the other hand, because of the weakness, suffering, social handicap, and early death, infanticide of the newborn has always reduced the Moon-child incidence in the Cuna population (*8*). The incidence was at its height a decade ago due to the influence of the great chief Nele Kantule, who said: "Because the Moon-children are on better terms with God, let us keep the Moon-children that they may tell us more about God." However, since the death of Nele Kantule, the incidence of Moon-children has declined, and although I have been told of 4 cases of infanticide of newborn albinos, usually the information never gets beyond the 3 old midwives who quickly bury the infant in the sand beneath the mother's hammock.

Albinism among the Cunas is inherited as a simple, recessive characteristic in our 1960 pedigree data. The percentage of albinos far exceeds 25 but, when adjusted to account for the Hardy-Weinberg considerations, these data fit very well with the expectancy, as shown in table 1.

At 1 degree of freedom in a chi-square table, a χ^2 of 0.870 indicates that the probability is between 0.30 and 0.50. This means that as great a deviation from the expectancy as present in our data would be expected due to chance alone in between 30 and 50 percent of all samples of the same magnitude. It would fit better were it not for infanticide.

Thus, our data on the inheritance of the Moon-child variation fit well with a mendelian 3:1 ratio. Hence we can say that the Moon-child variation is a simple, recessive mendelian characteristic dependent on one pair of mutant genes.

Heterozygotes are usually lighter in color than normal brown skins, and since this lighter color is preferred in this tribe there is actually a

TABLE 1.—Cuna Moon-child (albino) inheritance. Statistics to account for the Hardy-Weinberg law: 1960 field data

Number in sibship	Calculated percent of recessives (aa)*	Total No. of children	Calculated No.		Observed No.	
			Moon-children	Normal children	Moon-children	Normal children
2	57. 1428	22	12. 571	9. 429	15	7
3	43. 2432	48	20. 757	27. 243	18	30
4	36. 5714	64	23. 406	40. 494	18	46
5	32. 7784	75	24. 584	50. 416	20	55
6	30. 4128	30	9. 124	20. 876	11	19
7	28. 8512	14	4. 039	9. 961	4	10
8	27. 7813	24	6. 667	17. 333	9	15
9	27. 0295	9	2. 433	6. 567	1	8
Total		286	103. 581	182. 419	96	190

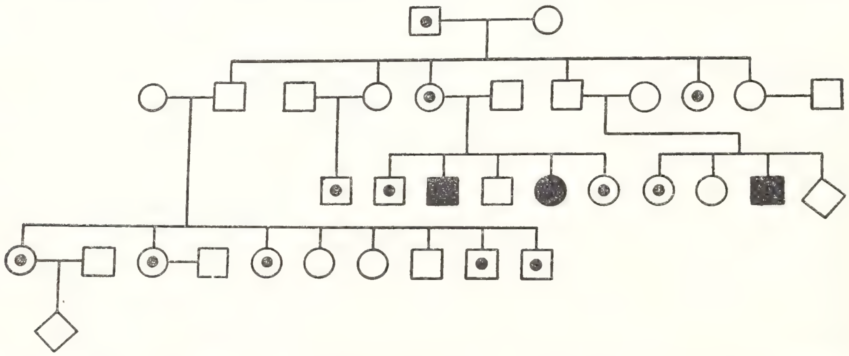
*The proportion of recessives (aa) is $\frac{0.25}{1-(0.75)^n}$ where n is the number of children in the sibship. Chi squared $= \text{sum of} \left[\frac{\text{deviations (observed} - \text{calculated)}}{\text{calculated number}}\right]^2 = \frac{57.471561}{103.581} + \frac{57.471561}{182.419} = 0.870$ with 1 degree of freedom $P = 0.30-0.50$.

social selection for heterozygote to marry heterozygote, guaranteeing the production of more albinos than would occur on the basis of purely random matings (text-fig. 1).

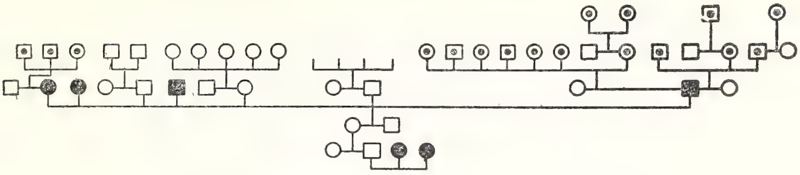
Heterozygotes for albinism were also lighter-skinned than the average among the Navajos (text-fig.2).

Although to speak of the dead is taboo in San Blas and to hide a lesion when one has it is customary, a number of years ago I began to realize that many of the Cuna albinos had open cancerous lesions on the cheek, eyelids, nose, lip, or arm in their teens and twenties. Further investigation revealed that metastases of skin cancer are probably the chief cause of death in albinos that live to grow up.

By inquiring quietly of educated Indians about these lesions of the Moon-children, I learned that a skin lesion became extensive, ran a clear exudate, and excavated inwardly. The patient took to his hammock, his arms and legs became emaciated, and after a number of months he



TEXT-FIGURE 1.—Pedigree of albinism among the Cuna Indians. *Squares* represent males; *circles*, females; *blackened symbols*, albinos; symbols bearing a *central spot*, light-brown-skinned individuals; *unshaded symbols*, normal dark-brown Indians.



TEXT-FIGURE 2.—Pedigree of albinism among the Navajo Indians. Symbols the same as in text-figure 1.

died. As the years passed, more educated Indians were willing to supply information. During the past year I have been advised of 5 deaths from skin cancer among Cuna albinos, and there were probably others.

The first biopsy specimen listed was examined by J. K. Sass, M.D., and the rest by A. W. McFadden, M.D. I have known personally for 12 years all 5 of the albino patients biopsied (fig. 4).

1) Pedro Arvila (albino Indian), 34 years—squamous-cell carcinoma had been removed from cheek: ulceration; temple. *Actinic keratosis.*
2) Galves Beker (albino Indian) 18 years: brownish-red papule; abdomen. *Solar elastosis.*
3) Ladislao Ismit (albino Indian) 10 years: brownish papule, slightly elevated; right forearm. *Verruca.*
4) Kawinti (brown Indian) 50 years: small ulcerated papule; suborbital. *Basal-cell carcinoma.*
5) Edna Perez (albino Indian girl) 14 years: dark-brown macule; right lateral forearm. *1. Ephelis. 2. Solar elastosis and hyperkeratosis.*
6) Placido Miseli (albino Indian) 24 years: papule; left subcostal. Slightly friable, rapid growth. *Elevated basal-cell carcinoma having a trabecular and a cystic pattern with the presence of mucous material occupying the cystic spaces.*

Recently, Dr. Archibald McFadden visited San Blas and has sent me a confirming account of his observations on the skin cancer of Cuna Moon-children that should be included here because of its brevity and completeness:

"All the skin cancer in the Cuna albinos is squamous cell (syn. prickle cell, epidermoid). The incidence of premalignant actinic (senile) keratoses in albinos is extremely high. These may be considered squamous cell carcinoma *in situ* or squamous cell carcinoma grade ½. With the exception of 2 or 3 children who were always protected from the sun, all albinos older than 3 years showed some actinic keratoses. These are firm, usually elevated, pinkish, almost translucent, asymmetric lesions commonly on the head, neck, and hands. Actinic keratoses usually have a dry 'warty' cap of keratin, and frequently become ulcerated c̄/ or s̄ mdg infection in adults. Ulceration without infection is good evidence for the clinical diagnosis of squamous cell carcinoma . . . . Metastatic carcinoma is frequently the terminal episode.

"Basal cell carcinoma occurs in normal Indians and rarely metastasizes. Therefore, basal cell carcinoma is not a killer and was not recognized in any albinos."

It should be stated that after this account was written, a basal-cell carcinoma was diagnosed in Cuna albino No. 6, Placido Miseli.

Linkage studies on albinism have revealed no associations, but if albinism is allelomorphic to red hair, as thought by Wright (*9*), it may someday be found linked to the A-B blood group series.

The incidence of albinism among Navajos is about 2 per 10,000; compared to that for Cunas (Moon-children), about 45 per 10,000; and for Europeans 1 per 10,000, as mentioned.

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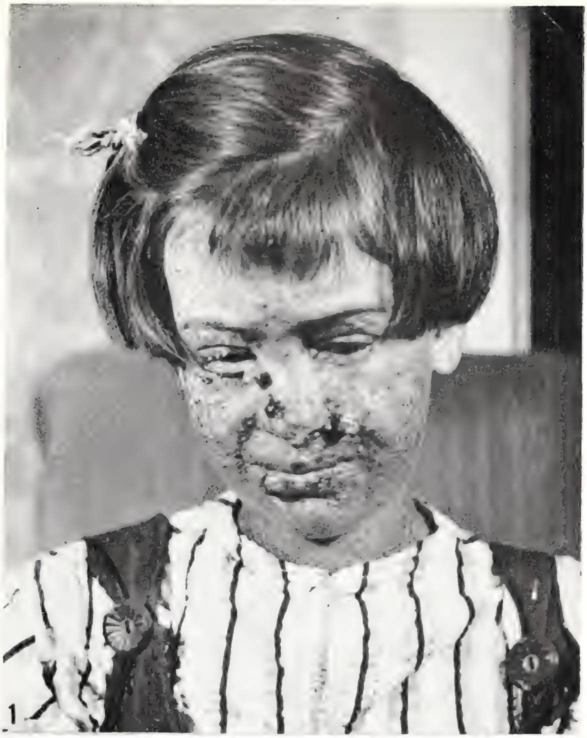
Plate 36

Figure 1.—Xeroderma pigmentosum in a 7-year-old girl of French Canadian extraction.

Figure 2.—Albinism in a 5-year-old Caribe Cuna (Moon-child) girl.



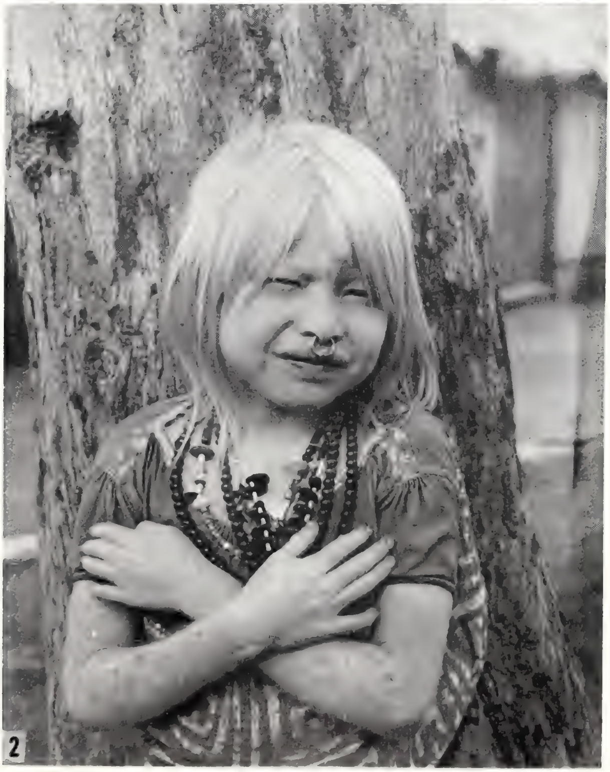


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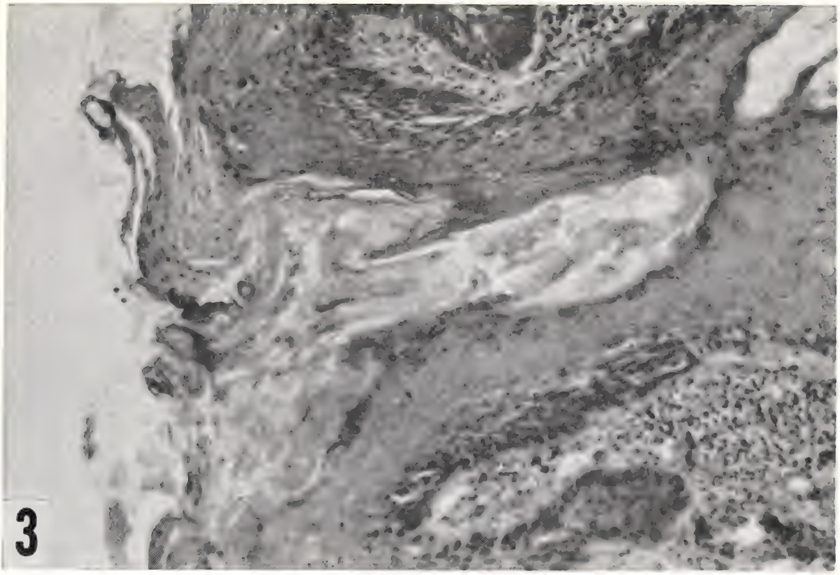
PLATE 37

FIGURE 3.—Section of xeroderma pigmentosum skin cancer. *Note* keratin plug, patchy parakeratosis with nuclei visible in the corneal layer, perivascular infiltration, thinning of the malpighian layer as well as irregular elongation of the rete ridges.

FIGURE 4.—Section of an albino (Moon-child) skin cancer. No. 6, Pacido Miseli. *Left end of section,* basal-cell carcinoma; remainder, normal tissue.



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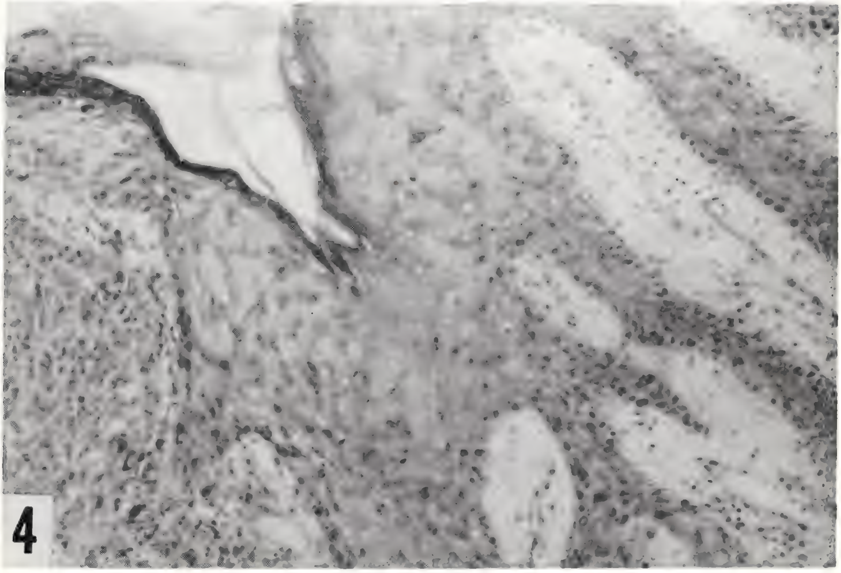

4

SPECIES DIFFERENCES IN PENETRATION AND ABSORPTION OF CHEMICAL CARCINOGENS[1, 2]

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SUMMARY

Species differences in carcinogenesis may be due to biochemical variations in the resistance of the target cells or to physical differences in the pathway through which the carcinogen reaches these cells. After painting with polycyclic hydrocarbons, the skins of different species and strains contained different amounts of the compounds. It is possible that genetic factors in mice may have caused a threefold range of concentration after identical painting procedures. In a few strains, significantly higher concentrations of hydrocarbons were found in the skin of males than in the skin of identically treated females. Hair growth was associated with slightly less initial uptake of hydrocarbons by mouse skin and with more rapid removal of the compounds from the skin. Benzo[*a*]pyrene penetrated the skin of dead mice readily, which suggested that active metabolic participation by the skin was not required for this process. High levels of carcinogen were found in the skins of treated mice, rabbits, and hamsters, whereas low levels were found in the skins of treated rats, guinea pigs, and men. These concentrations correspond to the reported sensitivities of these species after topical application of polycyclic carcinogens. Thus, differences in skin penetration may be a cause of species differences in skin carcinogenesis.—NCI Monogr. No. 10: 361-375, 1963.

SINCE THE earliest studies of experimental tumor production, it has been understood that different species respond differently. The diverse reports of work carried out with various species have raised questions regarding the validity of conclusions based on experimental work. Thus, any proposed mechanism of carcinogenesis must somehow explain species and strain differences which are often qualitative in nature. Further-

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[2] This investigation was supported, in part, by research grant C-5091 from the National Cancer Institute, National Institutes of Health, Public Health Service, and by the American Cancer Society, Inc., institutional grant IN-54A.

[3] This paper is dedicated to the memory of Dr. John J. Bittner, in whose laboratory I received some of my early training in cancer research.

I wish to acknowledge the assistance of Miss Mary Burnham and Miss Barbara Nestler who participated in much of the experimental work described in this report

more, extrapolation from experimental animal to human must be limited by the realization that, in many cases, not even extrapolation from rodent to rodent is possible.

Underlying the differences in response to experimental carcinogens are differences in the incidence of spontaneous tumors. Variations in tumor rates exist between species and between genetically distinct individuals within the same species. Such dissimilarities justify the general assumption that any exogenous agent stimulating neoplastic transformation will also exhibit a variable effect in different species or strains.

Causes of differences in response to exogenous carcinogens may be more complex that those causes in spontaneous tumor development. Two general mechanisms for species differences are likely. The first is a biochemical resistance of the cells of a species to induced neoplastic transformation. Such resistance might be general for all the cells of the animal or limited to a particular cell type. At the present time, the specific nature of this biochemical resistance to carcinogens is unknown. Nevertheless, genetic variations in spontaneous tumor development may be due to such factors. Studies of the Millers (*1*), Bonser (*2*), and Boyland (*3*) suggest that species differences in response to azo dyes and aromatic amines also may be due, in part, to biochemical factors.

A second cause of species variability is associated with anatomical relationships between the target cells and the site of carcinogen administration and between the target cells and neighboring tissues. Such variability might well be organ-specific, and in some cases might be amenable to prediction. The evidence presented in this paper suggests that the variable response of skin to polycyclic hydrocarbons is due to the influence of structure on the amount of carcinogen delivered to the ultimate target area.

Species differences in the neoplastic response of the skin are well documented. Before the polycyclic carcinogens were identified, it was well known that the rat and guinea pig would not develop skin cancer in response to coal tar painting (*4*). Comparable differences were later observed with pure hydrocarbons related to coal tar carcinogens (*5*). The experiments of Berenblum provide the best data (*6*). He found that 7, 12-dimethylbenz[*a*]anthracene (DMBA) was most potent in the rabbit and mouse, and less active in the rat and guinea pig. Skin tumors were produced in each of these species by this potent carcinogen, though the incidence in the guinea pig was low. Other studies, with less intensive methods with the rat and guinea pig, had given negative results (*5*).

An evaluation of the hazard of coal tar for human skin is difficult. This material, a very potent carcinogen for the rabbit and mouse, is almost certainly responsible for skin tumors which have appeared in exposed workers (*7*). On the other hand, crude coal tar and various extracts of coal tar have been used in clinical dermatology for many years. Under these rather circumscribed conditions, there is no convincing evidence that the material causes skin cancer in man. Nevertheless, it is usually concluded that coal tar is carcinogenic for human skin.

HAIR GROWTH AND STRUCTURAL DIFFERENCES IN SKIN

Among species, there are striking differences in the thickness of the skin, distribution of hair follicles, and growth patterns of the hair. The effect of hair growth on the action of topically applied carcinogens is profound. Chase and Montagna showed that epilation due to 3-methylcholanthrene (MC) was inhibited by hair growth (*8*). Workers in Copenhagen demonstrated that carcinogenesis was similarly inhibited when the painted skin contained growing hair (*9, 10*). The latter observation was confirmed and extended by Berenblum *et al.* (*11*).

Hair grows in waves in the mouse, rabbit, rat, and hamster. In these species, there is frequently an area of skin in which all the hair follicles are "resting." In the guinea pig the hair follicles grow in a mosaic pattern, so that active follicles are always found in any sizable area of skin. Human skin is also characterized by a mosaic pattern of hair growth (*12*). It is interesting that the species most sensitive to polycyclic hydrocarbons, namely, the mouse, rabbit, and hamster, all have a wave pattern of hair growth.

The thickness of the skin may also be associated with the resistance of species to carcinogenic stimuli. Thin skin is characteristic of the mouse, hamster back, and rabbit ear, whereas the skins of the rat, guinea pig, human, and the back of the rabbit are much thicker (table 1). One can speculate that a carcinogen, in order to produce a skin tumor, must penetrate through the superficial layers of epidermis to underlying cells. The path is of course long in thick skin but short in thin skin.

Regional differences in structure within an individual must also be considered in evaluation of the response to carcinogens. Regional differences in the weight of rabbit skin are evident in table 1. Another

TABLE 1.—Thickness of skin in various species

Species	Site	Number of animals	Number of specimens	Average weight of skin (mg/cm^3 ± SE)
Mouse	Back	10	10	26. 2 ± 2. 0
Hamster	Back	2	6	31. 7 ± 3. 2
Rabbit	Inner surface of ear	2	8	54. 9 ± 3. 0
	Outer surface of ear	2	8	111. 0 ± 13. 0
	Abdomen	2	8	167. 0 ± 12. 0
	Back	2	12	287. 0 ± 18. 0
Rat	Back	6	13	131. 0 ± 5. 0
Guinea pig	Back	10	10	185. 0 ± 6. 0
Man	Thigh	5	9	272. 0 ± 15. 0

striking regional difference in rabbit skin concerns the distribution and structure of the pilosebaceous apparatus. The dorsal skin contains large clumps of hairs, each having a single guard hair and very small sebaceous glands. The outer surface of the ear has more widely spaced hair tufts, but much larger sebaceous glands. On the inner surface of the ear, near the ear canal, guard hairs alone are found, and the sebaceous glands are very large and prominent (figs. 1, 2, and 3). These differences are of particular interest because the sebaceous gland is a major route for the penetration of polycyclic hydrocarbons into the deeper layers of the skin (*13–16*).

Because of these anatomical differences, it is of fundamental importance to determine whether the target cells of different experimental animals are exposed to similar or different levels of a carcinogen. Without such information, we cannot expect to understand the similarities and differences in the responses of species to these agents. As a step in the solution of this problem, the experiments described in this paper were undertaken to elucidate the nature of the penetration of polycyclic carcinogens into the skin.

Unless otherwise indicated, the data were collected according to standard methods which have been described earlier (*16*). Briefly, the animals were painted with 0.25 ml of solution delivered by an 0.25 ml tuberculin syringe to skin from which the hair had been clipped 24 hours earlier. The hydrocarbons were dissolved in benzene containing 1 percent heavy mineral oil. A 1 percent concentration of hydrocarbon was used. Under these conditions, the solution flowed rapidly over the entire clipped area of skin and left an even film of solution. In studies with the hamster, rabbit, and rat, and—except where otherwise mentioned—with the mouse, the skin was used at a time when the hair follicles were quiescent. Hair growth was determined in these cases by observation of skin thickness and by inspection for the presence of stubble 24 hours after clipping. Presumably, at least some of the hair follicles in the guinea pig and human skin were active at the time of study.

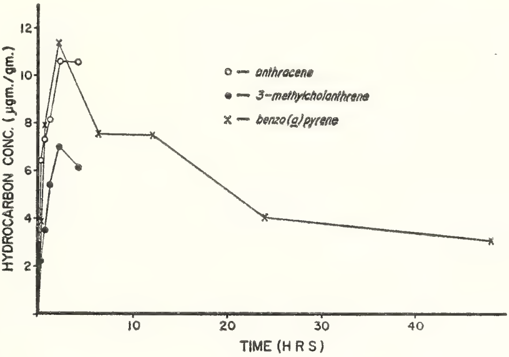
Two hours after painting, the skin was repeatedly scrubbed with benzene to remove surface hydrocarbon. The treated specimen was then removed, weighed, minced, hydrolyzed with alcoholic KOH, and extracted with benzene. The benzene extract was freed of water with anhydrous sodium sulfate and was then examined in a spectrophotofluorometer. Systematic errors in the method were controlled as described earlier (*16*). The variability of the specific measurements is indicated in the tables and charts.

RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND SKIN PENETRATION

Polycyclic hydrocarbons are lipides, virtually insoluble in water, moderately soluble in alcohol and acetone, and quite soluble in benzene.

Although they are aromatic compounds related to benzene, the complex ring structures of some create centers of chemical activity which are believed to be related to the carcinogenic potential of the molecules (*17*). Because they are soluble in fats, polycyclic aromatic hydrocarbons penetrate the sebaceous glands within minutes after skin painting, and most of the hydrocarbon found in the skin shortly after painting is in these structures (*14–16*). Hence it is not surprising that the cells of the sebaceous glands are quite sensitive to the attack of certain carcinogenic hydrocarbons (*18, 19*).

Maximum concentrations of hydrocarbons in mouse skin were found 2 hours after painting (text-fig. 1). The levels in the skin were, of course, due to two competing reactions: penetration of the compounds *into* the skin, and removal of the compounds *from* the skin by transport or by metabolic degradation. The relative magnitudes of these two reactions were studied by washing away the surface benzo[*a*]pyrene (BP) 30 minutes after painting. In this way, removal of the carcinogen from the cells could be measured in the absence of further penetration of the material into the area. The data showed clearly that transport or metabolism of the hydrocarbons was very slow when compared to the initial rate of penetration (table 2).

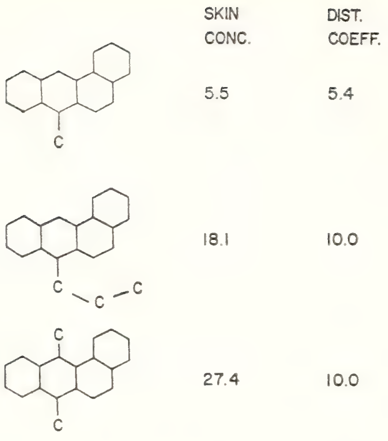


Text-figure 1.—Uptake and retention of polycyclic hydrocarbons by mouse skin.

From a comparison of the data in table 2 and text-figure 1, we may conclude that the curve obtained during the 1st hour mainly represents penetration of hydrocarbon into the skin, inasmuch as the rate of its

Table 2.—Loss of benzo[*a*]pyrene (BP) from washed skin

Time after painting (min)	Number	Average BP concentration (μg/g ± SE)
30	12	7.9 ± 0.7
60	12	8.9 ± 0.8
90	4	8.6 ± 1.5
120	16	7.9 ± 0.6
360	4	6.4 ± 2.8



Text-figure 2.—The relationship between the lipide nature of the molecule and the penetration into mouse skin. The distribution coefficient is the ratio of the concentration of the compound in the upper (nonpolar) and lower (polar) phases of a solvent system containing heptane, methanol, and water in the ratio of 5:9:1.

removal is slow. The fall in the rate of penetration after the first 30 minutes suggests that penetration of hydrocarbons into skin is favored by the presence of the volatile solvent. After the first few minutes, the solvent has largely evaporated from the surface, and the rate of penetration is low. At this time, the hydrocarbons are deposited on the surface of the skin as solid crystals suspended in saturated solutions of residual solvent, which consists primarily of the surface skin lipides and the mineral oil present in the original solution. This hypothesis explains the observation that a small amount of mineral oil in benzene, when used as a solvent for skin-painting experiments, favored the appearance of tumors and the penetration of hydrocarbons into the skin (*16*, *20*). It is also in accord with the report that, if a limiting concentration of hydrocarbon in solution was achieved, further increase in concentration did not promote greater penetration of BP into mouse skin (*16*).

The extent of the penetration of different hydrocarbons into mouse skin depended on the molecular structure of the compounds (table 3). Of 12

Table 3.—Effect of molecular species on concentration of hydrocarbons in the skin of A/St mice

Compound	Number of mice	Concentration in skin (μg/g ± SE)	Number of rings	Side chain	K*
Phenanthrene	38	96. 8 ± 5. 3	3	—	3. 1
7,12-Dimethylbenz[*a*]anthracene	16	27. 4 ± 1. 5	4	+	10. 0
Benz[c]acridine	8	22. 3 ± 1. 8	4	—	2. 6
7-*n*-Propylbenz[*a*]anthracene	8	18. 1 ± 2. 0	4	+	10. 0
7,9-Dimethylbenz[*c*]acridine	16	15. 2 ± 1. 0	4	+	4. 3
Anthracene	16	13. 3 ± 1. 5	3	—	3. 6
Benz[*a*]acridine	8	12. 5 ± 1. 3	4	—	0. 5
Benzo[*a*]pyrene	225	10. 7 ± 0. 3	5	—	5. 8
Benz[*a*]anthracene	14	9. 8 ± 1. 2	4	—	3. 8
3-Methylcholanthrene	44	8. 4 ± 0. 6	5	+	10. 4
7-Methylbenz[*a*]anthracene	8	5. 5 ± 0. 8	4	+	5. 4
6-Methylbenzo[*a*]pyrene	8	5. 2 ± 0. 5	5	+	6. 7

*K is the distribution constant of the hydrocarbon between the two phases (nonpolar/polar) of a system made up of 5:9:1 heptane, methanol, and water.

compounds studied, the ones which entered the skin in the greatest concentration had a small molecular size, both in "area" as indicated by the number of rings and in "thickness" as influenced by the presence of methyl side chains (*compare* 7-methylbenz[*a*]anthracene with benz[*a*] anthracene (BA), and 6-methylbenzo[*a*]pyrene with BP). A second characteristic apparently related to the degree of penetration was the relative solubility of the compound in polar and nonpolar solvents. This was demonstrated by the derivatives of BA, for which a high solubility in the nonpolar solvent was associated with high penetration, even though the molecular size was larger (text-fig. 2).

INTRASPECIES EFFECTS ON PENETRATION OF HYDROCARBONS INTO SKIN

It has been suggested that hair growth affects carcinogenesis by altering the rate at which the agents are removed from the skin (*11*). To study this possibility, we initiated growth of hair in mice by plucking all the dorsal hair when the follicles were quiescent. This procedure provided an area in which all the follicles were in well-defined stages of the hair growth cycle (*21*). The mice were used for the study at 4 days (anagen III), 12 days (anagen VI), and 22 days (telogen, or "resting" stage) after plucking. After painting, slightly more BP was found in skin with resting hair follicles than in that with growing follicles, but the differences were not statistically significant (table 4). Removal of the carcinogen from skin with growing follicles was somewhat more rapid than from skin with resting follicles. The differences in this experiment were small. Whether they could account for the difference in sensitivity of mice at different stages of the hair growth cycle is not apparent.

TABLE 4.—Uptake and retention of benzo[*a*]pyrene in mouse skin with active and resting hair follicles

Time after painting (hr)	Average concentrations of BP in skin (μg/g ± SE) of mice painted at:		
	4 Days after plucking	12 Days after plucking	22 Days after plucking
2	9.1 ± 1.3	8.5 ± 1.2	11.4 ± 1.2
6	6.4 ± 0.7	7.1 ± 1.1	7.6 ± 0.6
12	3.8 ± 0.6	9.6 ± 1.3	7.5 ± 0.7
24	1.1 ± 0.1	7.4 ± 0.8	4.1 ± 1.1
48	0.2 ± 0.1	1.4 ± 0.2	3.1 ± 0.4
96	0.0	0.0	0.0

There was a threefold range in the level of hydrocarbon in the skins of various strains of mice painted with BP (table 5). The absence of clear-cut stepwise differences between strains is of interest. If the differences were due to the predominant influence of only one or two gene loci, grouping of values about a few levels would be expected. The diversity of results suggests, instead, complex genetic control over skin penetration. Preliminary tests of F_1, F_2, and backcross offspring between the "high" JK and "low" A strains also support this conclusion.

TABLE 5.—Strain differences in uptake and retention of benzo[*a*]pyrene

Strain*	Number	Concentration of BP in skin (μg/g ± SE)	Strain*	Number	Concentration of BP in skin (μg/g ± SE)
DBA/1	24	33.7 ± 1.6	AK	16	19.6 ± 0.9
F	16	31.8 ± 1.5	C	8	17.4 ± 2.3
5 Prunt	24	30.4 ± 1.1	BRS	16	17.4 ± 1.4
JK	40	29.6 ± 1.1	C3H	32	15.6 ± 0.9
H	16	29.3 ± 1.7	L	16	15.2 ± 1.0
DBA/2	24	29.0 ± 1.2	CBA	16	15.1 ± 0.9
Pintail	16	28.8 ± 1.6	C57BL	32	14.9 ± 0.8
IPBR	16	27.8 ± 1.4	N	16	13.6 ± 1.0
2 Prunt	16	27.7 ± 1.7	CFW	16	13.1 ± 0.6
LaA	16	26.3 ± 2.0	CHI	16	12.9 ± 1.1
PBR	16	26.3 ± 1.8	I	16	12.4 ± 1.1
BRSUNT pb	24	24.3 ± 1.6	C58	16	12.0 ± 1.2
Low	24	22.0 ± 1.8	A	88	11.4 ± 0.5
C3HB	24	19.8 ± 1.0	ICR Swiss	16	10.2 ± 0.6

*DBA/1, DBA/2, and ICR Swiss mice were obtained from the breeding colony of the Roswell Park Memorial Institute, Buffalo, N.Y. All other mice were from the colonies of Dr. L. C. Strong, Biological Station, Springville, N.Y. The CFW and ICR Swiss mice are not inbred strains.

In other extensive studies there was no influence of sex on the penetration of several polycyclic hydrocarbons into the skin of A/St mice (*16*). Similar results were obtained with most of the strains listed in table 5. In 4 of the strains, however, the levels of skin BP after painting were significantly higher in males than in females (table 6). It is possible that this expression of a difference between the sexes may also be under genetic control.

TABLE 6.—Sex differences in uptake and retention of benzo[*a*]pyrene by mouse skin

Strain	Number of pairs	Concentration of BP in skin (μg/g)		P*
		Males	Females	
DBA/2	12	32.2	25.8	<0.01
BRSUNT pb	12	27.1	21.5	0.03
AK	8	21.6	17.6	<0.01
C57BL	16	16.3	13.5	0.04
N	8	16.4	10.8	<0.001
I	8	15.8	9.0	<0.001

*From an analysis of the differences between pairs of mice of the same age tested at the same time.

The mechanism of the genetic control over hydrocarbon localization could involve alterations of metabolic activity or changes in the physical pathways by which the compound reaches the target cells. In an attempt to decide between these possibilities, measurement of the penetration of hydrocarbon into the skin of dead mice was undertaken. A/St mice were painted at various times before or after being killed by cervical dislocation. The skin was removed for analysis 2 hours after painting, *i.e.*, at the time of the optimum concentration of hydrocarbon in living skin. The results indicated that, on the basis of wet weight, even more carcinogen was found in painted skin if the mice were dead (table 7). This was true even for mice that had been killed 24 hours before painting (26 hours before analysis). The difference has not been explained, but it is clear that penetration of the skin by BP does not require active metabolic participation by the skin.

TABLE 7.—Penetration of benzo[*a*]pyrene into dead skin

Hours dead before analysis	Average concentration of BP in skin (μg/g)*
0	7.5
2	9.6
4	12.8
26	15.6

*N_i=12; F = 8.04 ($F_{0.01}$=4.26).

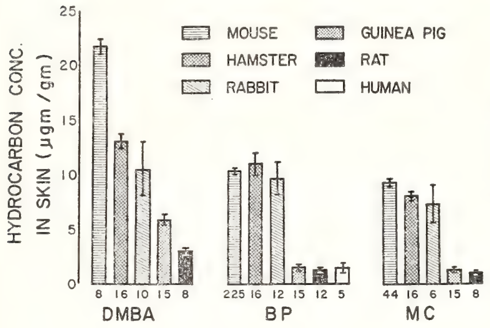
Other studies demonstrated that exposure of guinea-pig skin to ultraviolet light before or after painting does not affect the amount of hydrocarbon found. Likewise, exposure of painted mice to 1000 r of X rays does not affect the rate at which MC is removed from mouse skin (unpublished observations). These observations are of interest in view of the use of coal tar combined with ultraviolet irradiation in clinical practice.

INTERSPECIES DIFFERENCES IN PENETRATION OF HYDROCARBONS INTO SKIN

To compare the skins of different species, painting and analysis were separated by a constant 2-hour period. This time span gave optimal values for the mouse, guinea pig, rat, and hamster—the species in which sufficient time-series analyses were conducted. In a limited number of analyses of human skin, there were no significant differences in results obtained between 2 and 24 hours after painting. In most respects, the standard methods described earlier were used. Nevertheless, to provide larger areas of treated skin, 1.0 ml of solution was applied to species other than the mouse, in contrast to the 0.25 ml used for mice. In each case, the solution flowed rapidly over the area from which the hair had been removed, so that a very thin, uniform layer of solution remained on a specific area of skin. The increased volume thus served to expose a greater area rather than to provide a greater dose per unit of treated area.

The data show pronounced differences in the concentrations of hydrocarbon in the skins of the species tested (text-fig. 3). (The data from mice were obtained from the A/St strain, a stock with low BP uptake. Sprague-

Dawley rats were used. The remaining species were of undefined stocks.) The differences did not appear to be related to the thickness of skin, inasmuch as high values were found with the thick skin of the rabbit back. However, the species with skin resistant to the topical application of carcinogens were characterized by a low degree of penetration of the hydrocarbons. The results imply that species differences in carcinogenesis may be due, in part, to differential penetration of hydrocarbons into the skin. Cambel has reported that, in rats, there is an additional species-specific barrier to the movement of carcinogen from the sebaceous glands to subcutaneous tissues (*22*).



TEXT-FIGURE 3.—Concentration of carcinogen in the painted skins of various species. Skin from the human thigh and forearm was used, but dorsal skin from the other species.

In these comparisons, it is assumed that the analytical method provided a reliable measure of carcinogen actually available to the target area. Inasmuch as the amount of hydrocarbon delivered to the surface of the skin greatly exceeded that subsequently found within the skin, it was imperative to demonstrate that the washing procedure sufficiently removed the residual surface material. Fluorescent microscopy provided a satisfactory check of this question. It has been shown that, in mice, the washing procedure was effective, and that the bulk of the measured carcinogen was within the cells of the sebaceous glands (*16*). Parallel studies were undertaken with painted guinea-pig epidermis and appendages freed of dermis by incubation with collagenase (*23*). An examination of figure 4 showed that much of the BP in the preparation was within the cells of the sebaceous glands, but that a substantial amount was extracellular, within the lumen of the pilosebaceous canal. Accordingly, the concentration of BP in guinea-pig skin, indicated by text-figure 3, represents both intracellular and extracellular material. If it were possible to measure only the intracellular hydrocarbon, the level in the guinea pig would be relatively much lower than indicated in the figure.

It is apparent that some of the species differences in skin carcinogenesis are due to the differential exposure of the target cells. It would be of interest to determine whether species differences have a biochemical basis as well. This problem can best be studied by observation of the relative effects of doses of carcinogen which deliver the same amount of intracellular carcinogen to the target area. In this manner, any persistent species differences would be independent of the physical pathway by which the agent reaches the target. Studies of this type are currently under way in our laboratory.

Several other problems have been raised by the data. The experiments with dead mice indicate that penetration of hydrocarbons into skin does not depend on the metabolic activity of the cells. Accordingly, the variations between strains are probably due to chemical or physical differences in the skin structure. These could be related to the size and number of sebaceous glands or to the chemical nature of the cell membranes. The distribution and volume of the sebaceous glands can be measured histologically, and perhaps by chemical analysis. Results of such measurements would be of obvious importance in understanding skin penetration.

Still another problem raised by the experiments is related to the question of the carcinogenic potential of polycyclic hydrocarbons for man. The paradox raised by human experience in the clinic and in industry may be one of numbers only. In the survey made by Kennaway and Kennaway, more than 500,000 workers in selected industries formed a population prone to develop scrotal cancer (*24*). In this population, only 723 scrotal skin cancers were reported, an incidence of less than 0.2 percent. An extension of these numbers to clinical practice would explain the failure of dermatologists to record a significant incidence of iatrogenic skin cancer. Nevertheless, in a group of 5,274 chimney sweeps, 118 scrotal cancers were reported, an incidence of more than 2 percent. An incidence of this magnitude should be apparent were it to appear in practice. One might speculate that the chronic skin irritation experienced by industrial workers altered the permeability of the skin to the carcinogenic agents to which they were exposed. This possibility might also be tested in the laboratory.

In conclusion, it might be said that many factors affecting the response of experimental animals to skin carcinogens act through changes in the dosage directed to the target cells. These factors include the nature of the solvent, the concentration of hydrocarbon, the state of hair growth, and the species and strain of animal used. Inasmuch as skin painting has been used to study the potential hazard of human environmental agents, a knowledge of skin penetration is of importance.

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PLATE

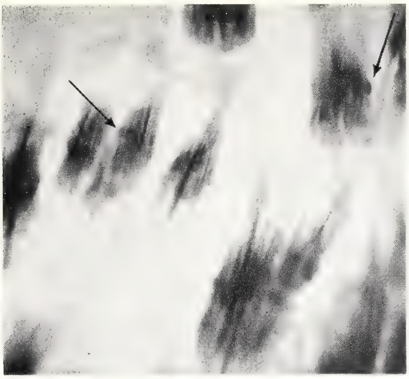
PLATE 38

FIGURE 1.—Wholemount preparation of skin from back of rabbit stained with Nile Blue. *Note* very small sebaceous glands indicated by *arrows*.

FIGURE 2.—Wholemount preparation of skin from outer surface of ear of rabbit.

FIGURE 3.—Skin from inner surface of ear of rabbit, near ear canal.

FIGURE 4.—Fluorescence photomicrograph of epidermis of guinea pig and appendages 2 hours after painting with benzo[*a*]pyrene.



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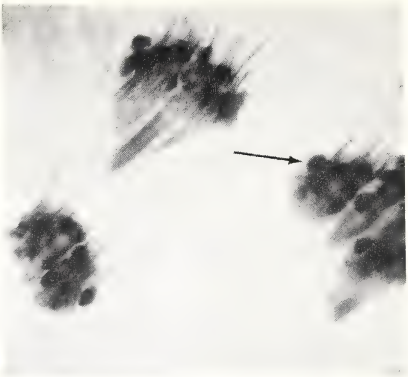

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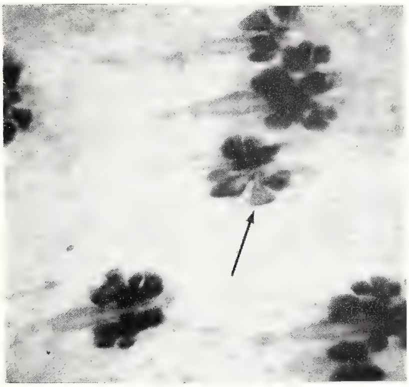

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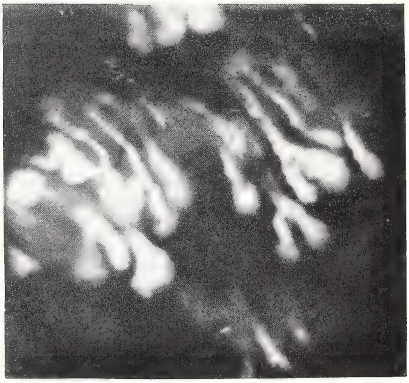

4.

CHEMICALLY INDUCED SKIN CANCERS IN MAN[1]

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SUMMARY

Chemically induced cancers of the human skin are historically of eminent importance in the development of human and experimental cancer research and also as they provided the first evidence on occupational carcinogenesis. They are, in many instances, part phenomena of chemospecific environmental cancer syndromes which are characterized by the hetero-organic occurrence of cancers. They are, therefore, of distinct significance not only for dermatologic but also for general medical, medicolegal, industrial, and public health reasons. The epidemiologic evidence at hand on chemically induced cancers of the skin strongly suggests that their actual frequency is much higher than that indicated by published records. A routine application of the information and guidelines provided by the traffic pattern of chemical cutaneous carcinogens in the human economy are a prerequisite for any future preventive control. The medical profession and public health agencies can make use of this information to discover these agents in the human environment, as well as the cancer hazards they create in different organs and tissues, in addition to the skin.—NCI Monogr. No. 10: 377–391, 1963.

AMONG THE various exogenous agents incriminated in the production of cancers in man, chemicals causing cancer of the skin occupy a special place for several reasons.

(*a*) Coal soot was not only the first environmental agent shown to induce cancer of any type in man but also the first to elicit cancer of the skin as well as the cause of the first recognized occupational cancer (scrotal cancer of chimney sweeps) [Pott, 1775 (*1*)]. The discovery of chemically induced skin cancers thus preceded by about a century the first claim concerning an actinic genesis of cancers of this organ system [Thiersch, 1875 (*2*); Hueper (*3–5*)].

(*b*) The carcinogenic action of the great majority of the many exogenous chemical carcinogens discovered during the subsequent 175 years was first demonstrated by their specific effect on skin [coal tar, pitch, creosote, anthracene oil, shale oil and wax, lignite oil and wax, petroleum oil, tar oils, asphalt, wax, carbon black and carbon, hydrogenated coal oil and tar (Bergius Process), arsenic]. It was shown later that under proper conditions of exposure some of these cutaneous carcinogens, *i.e.*, coal tar dust and fumes, pitch dust and fumes, petroleum oil mists and fogs, arsenic dust and solutions, may also elicit cancers in other organs and tissues, especially those of the respiratory and alimentary systems [Hueper (*6–9*)].

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., Apri '6-11, 1962.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

(*c*) The pre-eminence of chemically induced cancers of the skin in the field of environmental carcinogenesis is, moreover, attested to by the fact that the skin is the primary and often principal organ of contact and, therefore, the main target organ for many of these environmental chemical carcinogens. Chemically induced cancers of the skin furnish, for this reason, the largest contingent of all environmental, and particularly occupational, cancers attributable to carcinogenic chemicals [Hueper (*6, 10, 11*)]. Although the total number of skin cancers of chemical causation listed in published records comprises only some 6,000 to 10,000 cases, the actual number of such cancers is doubtless much higher. Chemical carcinogens acting on the skin are widely distributed in diverse industries and occupations containing large worker groups. These carcinogens are also constituents and contaminants of numerous and varied consumer goods, such as medicines, cosmetics, foodstuffs, sanitary goods, household goods, pesticides, and economic poisons [Hueper (*12, 13*)]. It is, therefore, reasonable to assume that an undetermined fraction of cancers of the skin occurring in such population groups as well as in the general population, and at present classified as so-called "spontaneous" cancers, owes its origin to chemical carcinogens.

(*d*) The epidemiologic, medical, and experimental evidence available in regard to environmental cancers justifies the additional conclusion that the occurrence of a chemical skin cancer hazard in a population group should serve as a warning that the exposure to the particular chemical carcinogen most likely also creates a cancer hazard to other organs. It is noteworthy that manifestations of polyorganic, chemospecific, carcinogenic syndromes among members of population groups having contact with cutaneous chemical carcinogens, such as arsenicals, coal tar, and paraffin oil, may be demonstrated not only in different individuals of such groups but sometimes also in the same individual where they take the form of multicentric and hetero-organic cancers [Hueper (*8*); Roth (*14–16*); Belisario (*17*)].

(*e*) It is, moreover, remarkable that the observation of chemically induced cancers of the skin has furnished the initial stimulus for the comprehensive experimental investigations on chemical carcinogenesis of recent decades, which started with the successful production of cancers of the skin in rabbits and mice by repeated and prolonged applications of coal tar, accomplished by Yamagiwa and Ichikawa (*18*) in 1915. The subsequent isolation and identification of specific chemical carcinogens of polycyclic aromatic hydrocarbon type from coal tar by Cook, Hewett, and Hieger (*19*) laid the foundation for later systematic chemical and experimental study of numerous synthetic, chemically related polycyclic aromatic hydrocarbons. Extensive search for their presence in various carbonaceous, carcinogenic materials, such as coal tar and pitch, petroleum products, shale and lignite oils, automobile exhaust, wood soots, and tobacco tars, also resulted from their work.

Cutaneous chemical carcinogens and chemically induced cancers of the skin have thus been fundamental factors in the development of modern experimental cancer research by providing a great mass of factual evidence and important information concerning the causation of cancers of various organs and tissues in man and animals by specific chemical carcinogens. The data derived from such studies have been useful in placing into proper perspective previously held concepts on carcinogenesis, such as the nonspecific chronic irritation theory, the theory of ubiquitous embryonic cell misplacements, and the hypothesis of a primary genetic and hereditary origin of cancer. The information obtained by a competent analysis of the significance of the numerous chemical and biologic observations made in chemical carcinogenesis, moreover, clearly demonstrates the distinct limitations confronting a general application of the more recently advanced virus theory of cellular cancerization.

(*f*) This outstanding position of chemically induced cancers of the skin in the field of environmental carcinogenesis receives additional support from the fact that these cancers, situated in the organ most accessible to direct inspection, reflect most fully the general image of environmental cancers and, therefore, may be considered their prototypes in etiology, epidemiology, diagnosis, symptomatology, pathology, and control. A critical presentation of the various observations pertaining to these aspects represents an important part in any discussion on chemically induced cancers of the human skin.

POLYETIOLOGY OF CHEMICALLY INDUCED CANCERS OF THE SKIN

In common with environmental cancers of other organs and tissues, those affecting the skin may be elicited by chemical and physical agents of different types. The polyetiologic principle of chemically induced cancers of the skin is established by the fact that the following chemicals are recognized carcinogens to the human skin: arsenic; incomplete combustion and distillation products of coal (tar, pitch, soot, tar oils, creosote, anthracene oil); distillation products of oil shale and lignite (oils, waxes, tar); hydrogenation products of coal (Bergius oils and tars); distillation and incomplete combustion products of petroleum and natural gas (coke, carbon, carbon blacks, asphalt, tar oils, fuel oils, lubricating oils and greases, crude paraffin oils, lubricating and cooling oils, diesel oils, exhaust fumes of diesel and gasoline motors, soot [Hueper (*20–22*); Eckardt (*23*); Henry (*24, 25*); Fisher (*26*); Page *et al.* (*27*); Combes (*28*); Jenkins (*29*); Scott (*30*)]. It is likely that many of the chemical carcinogens in the various combustion and distillation products of carbonaceous substances are identical, *i.e.*, that they belong to the groups of carcinogenic polycyclic aromatic hydrocarbons of the benzpyrene and benzanthracene compounds [Wolf (*31*)].

These chemicals have been demonstrated also in various vegetable tars and soots, which have been incriminated on the basis of mainly statistical and experimental evidence in the development of cancers of various organs, particularly the lung and stomach, but including also the skin. Cutaneous cancers have been observed after the prolonged medicinal use of tar ointments or lotions and after extended applications of creosote-containing hair preparations to the scalp [Berenblum (*32*); Hodgson (*33*); Rook (*34*); Belisario (*17*); Veiel (*35*); Büngeler (*36*)]. It is remarkable, on the other hand, that the very frequent and usually very prolonged exposure of the fingers of chain smokers to tars exuding from the tips of cigarettes has not resulted in cancerous reactions of the skin, although similar and usually occupational contacts with coal tar have repeatedly elicited such cancerous effects of the hands. Qualitative and quantitative differences in the chemical composition of different tars may readily account for marked variations in their carcinogenic potency.

There is, moreover, the distinct probability that part of the carcinogenic properties of combustion and distillation products of carbonaceous matter does not depend on the presence of the aforementioned polycyclic aromatic hydrocarbons, but seems to be related to the presence of other aromatic and aliphatic compounds, such as epoxides. Fractions of petroleum and shale oils free from demonstrable amounts of such polycyclic aromatic hydrocarbons have produced cancers of the epidermis when applied to the skin and subcutaneous tissue of mice [Berenblum and Schoental (*37*); Hueper, Kotin, Tabor, Payne, Falk, and Sawicki (*8*); Hueper and Cahnmann (*8a*)].

Whether carcinogenic carbonaceous combustion products formed during the acquisition of third-degree burns from tissue elements and retained within the scars may account for the frequent occurrence of cancers of the skin in such burned areas deserves consideration. This mechanism, together with prolonged exposure to soot formed by the burning of wood or charcoal, may also provide the specific chemical agents responsible for the development of cutaneous carcinomas in burn scars resulting from the use of personalized heating devices, such as kangri and kairo.

There is little valid evidence to sustain the claim that an occupational exposure to nitrates, such as that encountered by nitrate miners in northern Chile, is the chemical cause of cancers of the exposed skin observed among members of this worker group [Guzman (*38*); Prunes (*39*)]. Excessive solar radiation prevailing for such individuals represents the more likely causal agent. The exact chemical nature of the agents responsible for the cutaneous cancers in the region of the groin related to the wearing of the loin cloth, known as a dhoti among Indians, and for the penile cancers associated with prolonged exposures to smegma likewise remains to be determined.

Experimental evidence mainly obtained from mice that developed carcinomas after a cutaneous application of various chemicals used in the human economy suggests that occupational and environmental exposures to the following chemicals might create a cutaneous cancer hazard to man: mustard gas, β-propiolactone, urethan, and methylated naphthalenes. Of these chemicals, mustard gas is a known human carcinogen which when inhaled has caused cancers of the larynx and lung.

Finally, mention may be made of a recent medicolegal decision concerning the causation of a squamous-cell carcinoma of the hand in a German chromate worker who was awarded compensation because the cancer was considered as having developed on the basis of a chronic chromate dermatitis. This decision established a precedent, since "chrome holes" of the skin frequently found in chromate workers have not shown, according to general experience, any tendency to become cancerous and have thereby exhibited a biologic behavior similar to that displayed by cutaneous manifestations elicited by two other respiratory carcinogens, *i.e.*, asbestos warts and beryllium granulomas.

It is also remarkable that prolonged and intense cutaneous contact with any one of the rather numerous aromatic amino and azo compounds, such as dyes (yellow AB, yellow OB, oil yellow, auramine, brilliant blue, light green SF, butter yellow) and rubber antioxidants, naphthylamine, benzidine, and 4-aminodiphenyl, which have displayed a high carcinogenic potency for the urinary bladder and liver in man and/or animals, have not resulted, as far as it is known, in the development of cancers of the epidermis, though the carcinogenic aromatic amines are readily absorbed through the skin. Some of these carcinogenic chemicals were formerly incorporated in cosmetics used externally, such as lipstick, brilliantine, and hair pomade. The male-female sex ratio of 10 : 1 of cancer of the lip as well as the marked preponderance of labial cancers of the lower lip militate distinctly against any appreciable cancer hazard to the lips from such practices and agents.

SYMPTOM COMPLEX OF CHEMICAL CARCINOGENS

Since the skin is essentially the only organ accessible to ready, close, and repeated inspection and examination by physical, chemical, and pathologic methods, rather satisfactory information has become available on the various local changes preceding and accompanying the development of carcinomas of the skin induced by carcinogenic chemicals. Some of these tissue reactions form a part of the cancerization process and are, therefore, of "precancerous" nature, while others not related in any way to such cellular responses thus represent mainly significant and, to some extent, specific exposure stigmas. They may, therefore, be called "pericancerous" reactions; together they form the chemospecific carcinogenic syndrome. The cutaneous manifestations of these syndromes are commonly known as chronic arsenic dermatitis, chronic tar dermatitis, or chronic oil dermatitis. However, their etiologic specificity is limited since they show a marked overlapping in the types of symptoms elicited not only by different chemical carcinogens but also by the physical carcinogens, *i.e.*, with the symptomatic patterns known as chronic solar dermatitis and radio- or X-ray dermatitis. This phenomenon is well illustrated by the data listed in table 1, showing the correlations between individual pericancerous and precancerous cutaneous stigmas and the different chemical and physical cutaneous carcinogens. It becomes quite evident from these data that the body possesses, like with many other responses to physiologic and pathogenic agents, also in carcinogenic respects only a limited number of reaction mechanisms and reactions. The types or combinations of many of these reactions are not specific for any individual agent but are merely specific for groups of chemically, physically, or functionally related agents.

Apart from the fact that these syndromes are in many respects not really specific for the various chemical carcinogens, their occurrence is

TABLE 1.—Symptomatic environmental cancer pattern in precancerous and pericancerous reactions to environmental carcinogens

Exposure stigmas	
Reactions	Etiologic agents
Skin	
Alopecia	
Spotty loss of hair	Arsenic, ionizing radiations (radioactive substances, X radiation)
Atrophy	Arsenic, pitch, tar, asphalt, petroleum oils, radioactive substances, X radiation, ultraviolet radiation, solar rays
Skin grossly thinned and glistening in patches, associated with keratotic areas	
Eczema	Arsenic, asphalt, pitch, soot, tar mineral oils
Dry seborrheic patches on skin	
Keratosis	
Flat, discrete, scaly area on skin with raised pearly borders. Usually on parts of skin exposed to carcinogen, but may occur (with arsenic) in unexposed parts, particularly about sweat glands	Arsenic, anthracene, asphalt, creosote, crude mineral oil, paraffin, pitch, soot, tar, radioactive substances, ultraviolet radiation, X radiation
Hyperkeratosis	
Rough, fissured keratotic plaques with small, hard, wartlike horns usually on hands and soles. May become nodular and ulcerate	
Verrucae	
Hornlike hyperkeratosis	
Ulceration	
Breakdown of keratotic lesions	Arsenic
Chrome holes	Chromates, chromic acid
Leukoderma	
Patches of subnormal melanin pigmentation	
Leukomelanoderma	
Patches showing increased pigmentation and patches showing subnormal pigmentation of skin. Most common in areas of highest pigmentation and may involve oral mucosa	Arsenic, anthracene, asphalt, creosote, crude mineral oil, paraffin, pitch, tar, nonionizing and ionizing radiations, (radioactive substances, X radiation, ultraviolet radiation, solar radiation)
Melanoderma	
Patches of increased pigmentation	

not an obligatory requirement for establishment of the causation of a cutaneous cancer by any one of these carcinogens. Such symptomatic reactions may be quite fleeting and inconspicuous at times or may even be entirely absent as demonstrable precursor conditions of a cancerous development.

EPIDEMIOLOGIC ASPECTS

Although mortality data on skin cancer do not provide any representative information on relative skin cancer incidence rates for different population groups, they may show in a general way the existence of quantitative differences in the relative liability of members of different socioeconomic classes and of different industrial population groups to cancer of the skin. The evidence of Kennaway (*40, 41*) for socioeconomic classes (table 2) demonstrates that with a drop in the socioeconomic class there is an accompanying rise in the skin cancer mortality ratio. It is not likely that differences in the environmental or occupational exposure to carcinogenic solar radiation have been important factors in the production of these discrepancies in England, since it can be assumed that members of economically well-to-do classes may experience for various reasons (occupation, residence, sport, etc.) a higher exposure to ultraviolet rays than the socioeconomically poorer classes, though the latter have greater opportunities for exposure to carcinogenic chemicals for occupational and environmental reasons. The relative causal roles that chemical and actinic carcinogens exert in the determination of skin cancer mortality rate in rainy and foggy England are illustrated by the fact that the actinic

TABLE 2.—Standardized mortality ratio* at ages 35 to 65 in each social class from cancer of various sites

	Dicennial Supplements 1921 and 1931 [Kennaway (*40, 41*)]									
	Males									
	Social class and years									
	1921–23					1930–32				
Site	I	II	III	IV	V	I	II	III	IV	V
All sites	80	92	99	96	123	83	92	99	102	114
Lip	30	50	70	140	170	—	56	68	147	183
Tongue	48	73	95	100	165	78	60	98	110	143
Tonsil	25	88	94	106	163	(86)	76	93	97	147
Pharynx	89	78	100	160	161	(76)	83	99	87	130
Esophagus	76	91	104	88	130	74	87	98	94	130
Stomach	60	82	100	106	130	55	83	98	112	122
Upper alimentary canal	58	80	99	102	140	63	80	97	109	129
Lower alimentary canal	105	105	100	93	98	98	103	102	100	95
Larynx	72	96	93	96	135	60	81	98	90	143
Skin	63	73	100	120	150	(59)	75	95	116	133
Lung	100	109	97	79	124	107	96	101	91	112

*Registered percent of calculated deaths.

rate is about one tenth of that found in the population of sunny Australia, which is populated mainly by immigrants from England and their descendants [Belisario (*17*)]. England, on the other hand, has by far the largest number of recorded occupational cancers of the skin, which are attributed to contact with coal soot, tar, pitch, creosote, anthracene oil, shale oil, wax, and arsenicals.

Mancuso's (*42*) data on occupational skin cancer rates in Ohio (table 3) clearly reflect the solar influence in the rather high rates of skin cancer for farmers and those with outdoor occupations, while they indicate a predominantly chemical factor for those engaged in occupations that have contact with tar, oils, and arsenicals and show rather low skin cancer mortality rates.

TABLE 3.—Cancer deaths among males 15 years old and over in Ohio, classified by occupation and cancer site, 1947

Th. Mancuso (*42*)

	Lip, oral cavity, pharynx		Skin		Brain and central nervous system	
	Percentage of *all* deaths	Percentage of *all* cancer	Percentage of *all* deaths	Percentage of *all* cancer	Percentage of *all* deaths	Percentage of *all* cancer
Males 15 years and over	0. 60	4. 7	0. 32	2. 5	0. 21	1. 6
Service and manufacturing industry	0. 58	3. 9	0. 33	2. 2	0. 40	2. 8
Retail and wholesale	0. 71	5. 2	0. 24	1. 8	0. 28	2. 1
Agriculture	0. 32	2. 7	0. 57	4. 8	0. 09	0. 8
Construction	0. 69	5. 2	0. 48	3. 6	0. 30	2. 3
Transportation and communication	0. 76	5. 5	0. 18	1. 3	0. 13	1. 0
Iron and steel industry	1. 15	8. 0	0. 06	0. 4	0. 30	2. 1
Electrical and machine	0. 52	3. 4	0. 13	0. 8	0. 52	3. 4
Nonferrous metal industry	0. 40	2. 6	0. 13	0. 9	0. 00	0. 0
Mining and quarrying	0. 17	1. 3	0. 85	6. 7	0. 17	1. 3
Stone, clay, and glass	1. 10	10. 9	0. 44	4. 3	0. 00	0. 0
Rubber and plastic industry	0. 26	1. 8	0. 00	0. 0	1. 30	8. 9
Others and unknown	0. 59	5. 2	0. 29	2. 6	0. 11	1. 0

The available epidemiologic data for chemically induced skin cancers reveal the centrifugal spread and scatter pattern common to all industry-related environmental cancers. Cancer hazards start as a rule at the source of production in basic industries, progress through processing and consuming industries to workshops, merchandising establishments, trades, and finally reach private users of manufactured products and the general public. Primary contact with coal tar, for instance, occurs with those employed in coke oven and gas retort operations. It spreads from there to coal tar distillers, makers of coal tar paints and oils, makers of rubber, conduits of paper pulp, building materials, electrical appliances, patent fuel, and many other products. From this stage the hazard extends to road workers, makers of pharmaceuticals, masons, carpenters, painters, electricians, makers of optical lenses, and many other occupations that use such products. Finally, the hazard affects users of coal tar preparations for cosmetic and medicinal purposes and individuals inhaling coal tar fumes and dust contained in industrial effluents and in road abrasions.

A similar epidemiologic distribution pattern of cutaneous chemical carcinogens can be established for arsenic. It begins with the mining and smelting of arsenic-containing ores, extends from there to the production of arsenical insecticides, herbicides, feed additives and pharmaceuticals, passes then to the commercial use of these products, and ends with the consumption of arsenical pesticide residues in foods and beverages, tobacco products, and medicinal preparations, and with the inhalation of air contaminated with arsenic-containing industrial effluents and pesticides by members of the general population.

An adequate knowledge and a proper application of this environmental traffic pattern of chemical skin carcinogens in the human economy is a prerequisite to any competent and comprehensive epidemiologic study of chemically induced skin cancers.

The main cancer hazard resulting from an exposure to chemical carcinogens may be found at any link of this distribution chain, since it depends on the type and degree of contact prevailing at the various points of dissemination. When it occurs at the end of this chain, an epidemic-like type of environmental cancer may ensue. This event is perhaps best exemplified by the observation of arsenic cancers of the skin and other organs among consumers of drinking water polluted with arsenic in the Reichenstein region in Silesia and in the Cordoba region in Argentina, as well as more recently among German vineyard workers using arsenical pesticides and drinking wine contaminated with their residues [Roth (*14–16*)].

An adequate knowledge and a proper application of this environmental traffic pattern of chemical skin carcinogens in the human economy is essential to establish the role of these agents in the total panorama of skin cancers and their relative effectiveness at the various points of dissemination. Such information is, moreover, invaluable for devising proper protective and preventive measures for the control of cancer hazards related to these agents as they affect skin and other organs. The recently observed epidemic of occupational and dietary arsenic cancers among German vineyard workers indicates that the most intense and effective exposure to the chemical skin carcinogens does not necessarily exist at the source of production of such chemicals but may occur at the very end of the chain of distribution [Roth (*14–16*)]. Wherever the initial incriminating link is located in the chain of distribution of these carcinogens, it often furnishes, or should furnish, the base from which the entire spread of cancer hazards may be established.

The reported occurrence of cancers of the skin induced by carcinogenic chemicals in several members of the same family, such as chimney sweeps, indicates that a familial appearance of cancers of the same organ may not be due to any genetic factors but due to contact with the same or similarly acting chemicals. These may be from habitual or dietary sources within the home of such individuals or by their employment in the same trades. Contamination of work clothes with carcinogenic chemicals brought home for laundering also may create a cancer hazard for other members of the same household.

TYPES, SITES, AND INTENSITY OF EXPOSURE

With one exception, all chemical skin carcinogens exert their carcinogenic action by direct contact with the skin. The available evidence on arsenicals, on the other hand, indicates that their main cutaneous effect follows the ingestion of inorganic arsenicals. These are retained subsequently by sulfhydryl groups in the skin and are gradually excreted with its keratinous products (hair, nails, horny layer).

Observations on the topographical distribution of chemically induced cancers of the skin show that their sites are mainly determined by the duration and intensity of exposure to such agents in restricted areas, depending on the special type of contact sustained. This in turn is often determined by the type of occupational activities engaged in by the individuals involved. Workers handling coal tar and mineral oils therefore exhibit cancers affecting predominantly the skin of the hands, forearms, face and neck, while those employed in areas contaminated with pitch dust may develop cancers of the feet and legs. Special working conditions, such as those to which mulespinners, paraffin pressers, and chimney sweeps are subjected, predispose to the development of cancers of the scrotal sac in males or of the vulva in females as well as of the region of the groin. Arsenic cancers, on the other hand, show a preference for the nonexposed parts of the skin. Chemically induced cancers of the skin thus adhere in their topographic distribution pattern to the principles governing the dissemination of their actinic counterparts (table 4).

No reliable information is available on the minimum effective dose of any of the various recognized carcinogens that act on the human skin.

TABLE 4.—Topographical distribution of solar-arsenic and roentgen cancers of the skin

	Solar cancers (environmental)		Arsenic cancers		Roentgen cancers (occupational)
			Dietary	Medicinal and occupational	
Area of the body	Roffo (*43*) (%)	Tello (*44*) (%)	Tello (*44*) (%)	Neubauer (*45*) (%)	Hueper (*6*) (%)
Face, nose, neck	95.00	77.73	8.57	12.21	4.76
Hands, arms	3.07	1.43	10.70 { 6.27	46.61	84.52
Feet, legs	0.52	0.01	4.43		0.00
Haired parts of skin	1.02	21.33	80.73	41.18	10.72
Nonexposed skin	0.39				

Clinical experience, moreover, indicates that dark-pigmented individuals, such as Negroes, are less susceptible to the carcinogenic action of tars and oils than fair-complexioned individuals. It is, therefore, likely that any hypothetical minimum effective dose is smaller for whites than for Negroes, who, however, are not refractory. Presently available evidence on occupational and medicinal cancers of the skin does not support the view that males react more readily to chemical skin carcinogens than women.

Intensity and Duration of Exposure

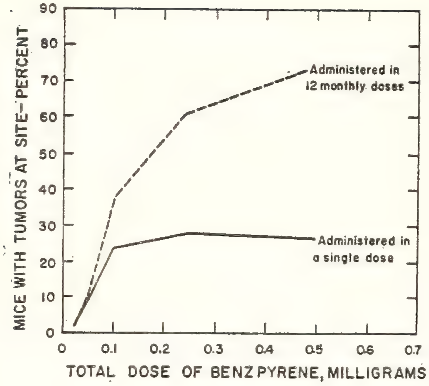
The duration of exposure to carcinogenic chemicals, which is followed by the formation of cancers of the skin, covers a wide range of time, extending from less than 1 year to over 50. The relative potency of the particular carcinogen, the intensity and duration of exposure to it, and the individual reactivity of the host are of importance here. General experience with occupational cancers demonstrates that the liability to cancer of the skin increases with the duration of exposure and may finally reach 100 percent of the population at risk [table 5 (*46*)].

TABLE 5.—Relation of exposure time to attack rate of pitch cancer of the skin [Sladden (*46*)]

Years of exposure	1–5	6–10	10–15	16–20	21–25	26–30	31–35	36–40	41 and over
Percent of pitch warts and cancers among exposed workers	17	21	36	67	69	71	77	80	100

Under the actual conditions prevailing during occupational exposures to cutaneous chemical carcinogens, a 100 percent attack rate of skin cancer has rarely been seen. In fact, the reported attack rates from occupational, medicinal, habitual, and dietary chemically induced cancers of the skin among members of the populations at risk have remained well below this rate in most instances. It is apparent from these observations that human populations seldom sustain exposures to chemical carcinogens comparable in degree with those resulting from an administration of the overwhelming doses of potent carcinogenic polycyclic aromatic hydrocarbons that have been applied to the skin of mice under most routine experimental procedures.

To be effective, the exposure does not need to be continuous. It may be, and often is, intermittent and may cease many years before the cancerous reaction becomes manifest, *i.e.*, at a time when it may be difficult to determine the time, type, and duration of exposure to a carcinogenic chemical responsible for the long-delayed appearance of the cancer. Observations made on man and experimental animals indicate that exposure to repeated but small doses of a chemical carcinogen is more effective in eliciting a carcinogenic response than a single exposure of the same total dose [text-fig. 1 (*47*)].



TEXT-FIGURE 1.—Production of tumors in mice with varying amounts of benzo[*a*]pyrene in single and repeated monthly doses: percent of mice with tumors and total dose.

Latent Period

The length of the latent period, from the start of an exposure to a carcinogen until the appearance of a malignant lesion, is often identical with the length of the exposure period, especially under occupational conditions. Since it depends on the relative potency of the carcinogen, its intensity of contact, and the individual susceptibility of the person exposed, the latent period in all types of chemical skin cancers varies widely, both for the same and for different chemical carcinogens (table 6).

TABLE 6.—Latent periods of cancer of the skin caused by chemical carcinogens

Agent	Average latent period (years)	Range of latent period (years)
Arsenic:		
Medicinal	18	3–40
Occupational	25	4–46
Coal tar	20–24	1–50
Creosote oil	25	15–40
Mineral oil	50–54	4–75
Crude paraffin oil	15–18	3–35
Solar radiation	20–30	15–40
X radiation	7	1–12

Whenever there is a lag between the end of the exposure time and the beginning of the latent period, pericancerous and precancerous manifestations may, but do not necessarily, exist during the period that precedes the advent of cancerous developments. The lag period sometimes may extend over many years before the chemical cancer makes its appearance and may thereby obscure the demonstration of an exposure to a chemical carcinogen that has occurred years, or even decades, before.

The age distribution of persons with occupational cancers of the skin of both chemical and actinic origin reveals for most agents a shift toward younger age groups than those found for skin cancers of unknown etiology (table 7). This shift is due in part to the more intense exposure to powerful carcinogens prevailing under occupational conditions, and in part to the fact that the age at which such cancers become manifest depends also on the age at which the first contact with the carcinogen occurs. A pertinent illustration of these influences on age distribution is presented by experiences reported from England concerning the average age associated with the occurrence of scrotal cancers among chimney sweeps before and after the turn of the century. The difference observed is attributable to the fact that the age of entrance into this occupation was distinctly lower before 1900 than after (table 8). Similar and confirmatory observations on the dependence of the average manifestation age of occupational cancers of the bladder on the age at entrance into the production of carcinogenic aromatic amines were reported by Goldblatt [table 9 (*48*)].

TABLE 7.—Age distribution of persons with occupational cancers

	Years of exposure												
	10–30		31–40		41–50		51–60		61–70		71 and over		
Organ and agent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Total number
Skin													
Arsenic	10	8.7	28	24.3	35	30.4	24	20.9	17	14.8	1	0.9	115
Pitch	6	5.1	14	12.2	34	29.6	38	33.1	20	17.3	3	2.7	115
Tar	—	—	6	7.5	14	17.5	15	18.7	32	40.0	18	16.2	80
Paraffin	—	—	6	5.9	30	28.8	38	34.8	29	27.6	3	2.9	106
Shale oil	—	—	12	17.8	22	32.3	23	33.8	6	8.9	5	7.3	68
Solar radiation	2	0.9	8	3.6	24	10.7	58	25.8	76	33.8	56	25.0	224
X radiation	—	—	18	51.4	9	26.1	6	17.1	0	0.0	1	2.9	35
Control	13	1.8	74	10.4	125	17.6	165	23.2	174	24.5	157	22.2	709

TABLE 8.—Age distribution of chimney sweeps afflicted with scrotal cancer

Age range	1892 (number of cases)	1935 (number of cases)
25–35	4	1
36–45	7	6
46–55	14	18
56–65	4	33
66–75	—	28
76–85	—	17
Average age	45–50	61.9

TABLE 9.—Relationship between age at entry into industry, diagnosis, and death of persons with aromatic amino-cancers of the bladder [Goldblatt (*45*)]

Age at entry into industry	Under 30	30–40	Over 40
Number of cases	40	17	9
Number of deaths	10	5	4
Average age at diagnosis of cancer	42	51	58
Age range	25–52	41–62	47–66
Average age at death	44	54	66
Age range	35–41	51–60	61–69

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PENETRATION AND ABSORPTION OF IONIZING RADIATIONS IN SKIN [1]

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SUMMARY

The penetration and absorption of ionizing radiation in the skin lead to generally predictable effects, including destructive effects which can be roughly quantitated, but subject to biologic and physical variations. Current concepts provide that all forms of ionizing radiation have a similar end result for equivalent energy transfer. Carcinoma occurred in cases of severe skin damage due to X radiation (30% in several large series) and due to radium exposures. Other forms of ionizing radiation have been shown to have carcinogenic capability in the skin, but without sufficient data in man to provide a clear pattern. Animal data have been extremely useful, and comparable in certain limited aspects, including probably that of carcinogenesis, but as in other biologic situations have not been directly translatable to man. Prolonged latent periods in some of the more recent forms of radiation probably have not yet occurred.—NCI Monogr. No. 10: 393–406, 1963.

THE RELATIONSHIP of ionizing radiation to carcinoma of the skin is a generally recognized one, in spite of a remarkable lack of identification of the initial and intermediate processes involved, the dose relationships, and the nature of varying responses in man and animals. This discussion is directed toward a consideration of the physical factors generally encountered together with certain of the limited morphologic evidence available. Among the biologically oriented observers there is often an exaggerated impression of the precision to which the physical factors may be measured or predicted. This is perhaps accentuated by the variability that exists in the response of biological structures, the rather inadequate means of measuring biologic response, and perhaps, as has been stated by many observers, the rather limited manner in which biologic structures may respond to what is basically some degree of destructive energy. In some responses to radiation there are rather distinctive patterns in tissue that may be linked with physical factors. More often, as with the more fundamental aspects of carcinogenesis, the relation with the physical factors is obscure.

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PHYSICAL FACTORS

Certain basic factors are of interest in the physical field:

a. degradation characteristics of the particle or electromagnetic energy form within tissue
b. attenuation and general absorption characteristics
c. relative absorption of the various energy forms by tissue components
d. the ultimate physical effect at the cellular level, which culminates in ionization and probably excitation

These aspects are not independent, and all might be grouped and described as the characteristics of energy transfer within tissue. Unfortunately the means for full understanding of this process in tissue are not at hand, nor have model systems been formulated as adequate substitute, though certain aspects have been clarified and will be mentioned in the following specific physical energy forms. The specific subject of interaction of the several forms of ionizing energy at cellular and molecular levels is not within the scope of this discussion.

ALPHA PARTICLES

From the standpoint of skin effect, these poorly penetrating particles present a very limited problem. Five Mev α-particles penetrate approximately 45 μ within tissue. Naturally occurring α-emitters have an average range of tissue penetration of approximately 35 to 100 μ in tissue. A recent study by Witten *et al.* (*1*) has clarified some aspects of α-particle effect on the skin. By the utilization of polonium^{210} (5.3 Mev) in the form of a plaque applied to the skin, erythema and subsequent pigmentation were regularly produced. In tissue equivalent studies the same authors determined that the dose at a depth of 40 μ is 0.001 percent of that at the surface. They further determined the thickness of the epidermis in 2 normal subjects to be 30 to 75 μ and concluded that most α-particles are unable to penetrate the epidermis. This creates some doubt as to the effectiveness of this form of radiation when applied to the skin and by the same token the ability to cause damage. Thorium X, which has been used clinically for many years, has not produced serious late sequelae such as ulceration or malignancy (*1*). Other physical factors, of importance in the interaction of α-particles and tissue at the cellular level, are obviously less applicable to the inactive, superficial cellular components of the epidermis, which serve merely as an absorbing and shielding substance. One factor that may be significant relates to the liberation of energy or the ionization pattern of the α-particle, as described in the phenomenon of specific ionization. While related to energy of the particle, ionization increases as the particle loses velocity and results in the liberation of a significantly larger portion of the energy near the terminal part of its path. This has particular significance in α-emitters applied to the skin, which tend to enhance the effect at the basal layer and dermal papillae. An unusual aspect of α-particle exposure of the skin is revealed in studies of

Lippincott *et al.* (*2, 3*) involving the neutron α reaction of thermal neutrons on boron present in the tissue by intravenous administration in pigs. Lithium[7] and some component of thermal neutrons, representing the action of α-particles show extensive degenerative and destructive effect of skin and subcutaneous tissue. Ulceration and vascular endothelial response of an advanced degree were present, with squamous-cell carcinoma at an ulcerated area in 1 animal.

BETA PARTICLES AND ELECTRONS

This form of radiation has assumed greater importance with the advancements in design and application of electron-accelerating equipment, and the greater opportunities for contact with β-emitting radioactive materials. Studies have been carried out to clarify the effects of β-radiation, the most definitive being related to the skin. Low-Beer (*4*) presented detailed studies with phosphorus^{32}. Zirkle (*5*) edited an extensive series of reports of external effects of phosphorus^{32} which included the late change of carcinoma in mice and rats. Moritz and Henriques (*6*) reported the first comprehensive study combining morphologic and physical elements, utilizing a series of isotopes of varying β-energies on the skin of pigs. They provided useful data on threshold and reversibility for acute and chronic changes, with reference of all doses to calculated values at the basal layer. Lushbaugh, Storer, and Hale (*7*) described β-radiation effect in the skin of the rat with emphasis on the reparative processes. Shubik *et al.* (*8*) have shown a latent tumor effect in β-irradiation followed by croton oil. Studies in humans have been carried out by Griffith *et al.* (*9*) in individuals engaged in radon preparation with a description of atrophic changes without carcinoma. Teruoka (*10*) and Koyama *et al.* (*11*) report the changes in the Japanese fishermen who were exposed to radioactive fallout but subsequently recovered. More detailed and extended observations on the Marshallese exposed to radioactive fallout at the same time have been reported by Conard *et al.* (*12–14*). Recent observations (*15*) indicated residual pigment alterations, atrophy, and scarring, but without gross evidence of breakdown or premalignant change. An increase in pigmented moles was suggested in the involved areas in several cases. Current observations (*16*) are similar. Additional observations have been made in accidental exposures by Knowlton *et al.* (*17*), Conard and Tessmer (*18*), Cramer *et al.* (*19*), Robbins *et al.* (*20*), and Hempelmann *et al.* (*21*). Long-term effects after exposure to radioactive fallout from a morphologic point of view have been reported by Tessmer (*22*) as well as the first case of malignancy in an area of skin damaged by radioactive fallout (*23*). Davis *et al.* (*24*) have provided experimental data on electron exposures (1.5 Mev). Schubert *et al.* (*25*) report carcinoma from local β-radiation from subcutaneously implanted strontium^{90} glass beads in rats, at levels of radiation in one instance too low to produce epilation. The rate of tumor formation was proportional to the logarithm of radiation intensity. Further evidence of tumor formation in the rat has been provided by Albert, Newman, and Altshuler (*26*). Their data indicated different dose relationships for two general types of tumor of the skin. Adnexal-type tumors (hair follicle and sebaceous) predominated at intermediate levels (4000 rad) while epidermoid carcinomas were more common at higher levels which produced severe skin damage (7–10,000 rad).

β-Particle and electron irradiation of the skin as a reasonably well-defined entity has not progressed to full analysis. Carcinogenesis is established in the mouse, rat, and rabbit (*26–28*). Long-term lesions have been described in larger animals and in humans, but the only indication of carcinoma has been the report of malignancy in an area of bovine skin after damage by radioactive fallout (*23*). In addition to certain qualifications this is a recent finding, and a necessary interval may not have occurred in other exposures. In addition to limited use on the skin a much more effective degree of control exists in more recent years in the case of β-particle and electron exposures as compared, for example, with X rays. For this and other reasons the exposure to this form of radiation has been relatively small and the number of damaging exposures particularly limited.

The physical basis of β-particle and electron effect in tissue has not been fully clarified. No practical form of spectral or depth measurements is available. The β-particles of radioactive materials are emitted as a range of energies, which may be characterized by a single constant maximum value, but more usefully by the average of the energies. Dosages at various depths of penetration (from the skin surface) then represent also a range with presumably a single relatively ineffective maximum. The range for effective biologic action is generally considered to be two thirds of the maximum range (*29*). A uniform tissue equivalent phantom may indicate this relation, but methods of direct histologic observation of tissue are neither sufficiently sensitive nor specific to contribute much definitive information beyond certain characteristic combinations of physical and histologic data (*22*). Also some correlation with acute findings and chronic change other than carcinogenesis has been achieved, with doses calculated at the basal layer (*6*). In the special circumstance of monoenergetic electrons, penetration and energy liberation in tissue may be characterized more adequately, approximately 0.5 cm penetration in tissue per Mev, though some exception has been noted between physical data and tissue depth response (*30*).

In some respects the β-particle and electron effect may be considered the fundamental one, based on the current concept that all penetrating ionizing radiation transfers energy in tissue by the production or excitation of electrons.

GAMMA AND X IRRADIATION

This form of ionizing radiation is the most widely encountered and most significant from its over-all effect. The energy transfer pattern to the skin, compared to α- and β-particles, makes the circumstances of exposure markedly different. Only the low energy X and γ- rays are absorbed in the skin to any significant degree, and filtration and higher energy sources have tended to reduce the limiting and undesired relative skin dose. The point of maximum energy transfer in high voltage therapy is not at the skin surface but at some point below.

The general categories of γ- or X-irradiation sources in relation to skin damage and potential carcinogenesis may be stated as:

a. long-term repeated exposures of an occupational or diagnostic nature, *e.g.*, fluoroscopy
b. deep therapy with skin absorption
c. superficial therapy for skin effect
d. accidental exposures to low energy radiation, *e.g.*, fallout
e. experimental exposures

All these categories have their recognized and occasionally unrecognized sources of radiation with no demonstrable difference in reactive pattern when dose and location of energy transfer are considered.

Gamma and X-radiation effects of the skin have been extensively documented from the radiodermatitis observed soon after the discovery of X irradiation (1896) and the first case of carcinogenesis (*31*). The classical morphologic descriptions of the tissue alterations by Wolbach (*32*) and the comprehensive survey of the effects of radiation on normal tissues by Warren (*33*) have been particular landmarks. Carcinoma is a recognized late development in X or γ-irradiation of the skin. Robinson and Masters (*34*) reported a 29.5 percent incidence of malignancy in a series of radiation injury cases requiring surgical management. Leddy and Rigos (*35*) reported 39 cases of carcinoma in 135 cases of radiodermatitis or 29 percent. Less frequently sarcomatous types of tumors have been reported after radiation in experimental animals and humans (*36*, *37*).

A special segment of the X-ray spectrum, grenz rays (10 to 25 kv), has been utilized for superficial therapy. Absorption in the skin is relatively complete, without the undesired effects in deeper tissues. Neither the portion of the spectrum nor the biologic effects appear to have a unique character in comparison to other ionizing radiation when the site and amount of energy transfer are considered. Shapiro *et al.* (*38*) reported the carcinogenic effect of grenz rays in mice.

NEUTRONS

This form of radiation is a relatively uncommon one with less biological data, particularly for the skin. From the physical standpoint neutrons consist of particles with unit mass, without charge, and not ionizing in the same sense as α, β, and γ radiation. The mechanism is neutron capture mainly by low atomic number nuclei, particularly hydrogen and nitrogen with subsequent energy release by ionizing forms. Physical data indicate relatively low absorption and energy liberation in the skin for fast neutrons (7–8 % of incident dose) (*39*).

Ely *et al.* (*40*) have reported in detail the pathologic effects of chronic neutron irradiation by fast neutrons up to approximately 500 r equivalent (n) over a period of 1 year. The only findings referable to skin were alopecia in rats and graying accompanied by alopecia in dogs. One rabbit tumor was found, which was considered to be an amelanotic melanoma, though the primary site was not identified. With significant pathologic findings in other systems and the known absorption characteristics, the limited response of the skin may be considered consistent with the relatively low energy transfer of fast neutrons in the skin.

Stone has reported the effects of fast (16 Mev) neutrons, 110 n, producing minimal erythema as compared to 650 r of 200 kv X ray, with the observation however that the relative effect was greater for late manifestations (*41*).

Thermal neutrons, which form a special case in terms of increased capture and liberation of γ-energy, have limited practical application in skin and little experimental data are available. Lippincott *et al.* (*2*) reported the morphologic changes in the skin of pigs after exposure to thermal neutrons as a portion of studies related to the boron-capture therapy. Tissues received 5×10^{12}n per cm^2. Minimal erythema occurred in 24 hours, with no further changes grossly or histologically at the time of examination ranging from 15 to 112 days.

In considering the tolerance to thermal neutrons, Capron *et al.* (*42*) indicated that the ionization dose passes through a maximum 0.3 cm from the surface and at significantly higher levels than previously determined.

The most informative data on the effects of thermal neutrons on the skin have been provided by Yamamoto in conjunction with studies on boron-capture therapy (*43*). The rabbit ear will tolerate 10×10^{12} thermal neutrons per cm^2 with findings limited to edema, temporary epilation, pigmentation, and superficial desquamation. The circumstances of exposure included an approximately equal rad contribution in γ-radiation as inherent contamination. Of particular interest in this regard is the RBE (relative biologic effectiveness) for thermal neutrons as compared to 250 kvp X ray, with figures of 3.4 reported by Bond *et al.* (*44*) in the mouse and 3.9 by Yamamoto in the rabbit (*43*).

HEAVY PARTICLES

Heavy particles include protons, α-particles, and other heavier atomic structures. These may be encountered under conditions of natural or artificially radioactive materials, artificially accelerated particles, or as a component of cosmic radiation environments.

Falkmer *et al.* (*45*) reported the effects of approximately 40 Mev protons (from a synchrocyclotron) on the skin, with comparative data from 220 kv roentgen rays. They concluded that the changes in the skin were qualitatively the same, with slightly less skin damage by protons for equivalent doses. While these investigators utilized protons at their point of maximum energy release, the so-called Bragg peak, by the device of interposing 17.6 cm of Lucite (approximately tissue equivalent), it is to be pointed out that the skin would otherwise have received 10 percent of the surface dose as compared to approximately 45 percent. These observations were limited to short-term effects.

The skin provides one of the indexes of heavy particle effect by the changes observed in hair pigmentation. When exposed to heavy primary cosmic radiation, C57BL mice and guinea pigs showed focal depigmentation of hairs in a number of instances with linear configuration indicating the path of a heavy particle (*46*, *47*). The exposed group of 96 mice showed no evidence of a significant difference in carcinogenesis between exposed and controls.

COMBINED RADIATIONS

The nature of many radioactive sources is such that combinations of the foregoing energy forms occur, at times in fixed and predictable relationships, at other times beyond practical dosimetry or calculation. In some cases, as in radioactive fallout, an appreciable variation occurs in both intensity and spectrum. Neutron exposures are accompanied by some proportion of γ-ray level. These circumstances produce some complication in dosimetry. In certain relatively unusual experimental conditions monoenergetic radiation may be utilized. As has been stated, the tissue effects at the time of interaction and energy transfer are regarded as identical for all forms of ionizing radiation.

RELATIVE ABSORPTION OF IONIZING RADIATION IN SKIN

Coupled with the foregoing factors, it may also be important to view the possible variation in energy liberation due to the differential absorption of the various tissue components in skin. This is a consideration apart from the regional effects due to the penetrating characteristics of the particular form of radiation. There is relatively uniform absorption at energies of 0.2 to 10 Mev, with significant variations at lower and higher energies, though the data are available only for bone, muscle, and fat (*48*). Based on well-recognized differences in density, demonstrated for example in low-energy microradiographs, certain significant differences in energy absorption may occur. This may form a part of what is generally considered tissue sensitivity.

RELATIVE SENSITIVITY OF TISSUE COMPONENTS OF SKIN

One important phase of the analysis of skin changes is the relative sensitivity of the various components. This must be immediately qualified by the so-called indicators or the methods by which such sensitivity is determined. Morphologic criteria have been utilized, but are somewhat unsatisfactory, particularly in the detection of early changes. An outstanding example is the advanced change which may be observed in the elastica in later periods, while little change is noted at earlier intervals except in exceedingly high-dose levels. The earliest morphologic response may not necessarily be an indication of the most sensitive tissue. Functional changes may be more effective to indicate radiation effects, such as the well-established increase in permeability of the vascular system after radiation (*49*) or the relationships between response to radiation and deoxyribonucleic acid turnover as indicated by Hevesy (*50*). For practical purposes the structures of the skin may be listed in the following order of sensitivity:

a. basal-epidermal tissues
   vascular endothelium
   melanocytes
b. hair follicles
c. sebaceous glands
d. arrector pili muscles
e. sweat glands

The matter of tissue sensitivity is a complex one, with perhaps the most definite relationships with the mitotic cycle. The increased sensitivity of cellular elements to radiation during the early portion of the mitotic phase has been reported (*51*). This coupled with the graded response of mitotic activity to radiation (*52*) gives basis for the concept of the direct relationships between "tissue activity" and radiosensitivity.

SKIN PROTECTION

Some consideration in a discussion of the carcinogenic action of ionizing radiations should be given to the protective measures which have been reported, for such insight as they may provide. Protection from acute or apparent damage to the skin may not be equated with carcinogenesis on the basis of any clear experimental evidence. Fogh reported more than doubled skin tolerance by the local application of cysteamine and *p*-methoxythiophenol in the rabbit, with hairs reappearing after doses up to 13,500 r (*53*). Houghton *et al.* (*54*) effected a modification and delay in the necrosis of skin after radiation by parenteral cortisone, with the prevention of deep ulceration and scar contraction. Forssberg obtained protection against radiation by cysteine injection subcutaneously (*55*). Ungar *et al.* (*56*) reported a protective effect in the form of reduction of ulceration in rats receiving spleen homogenate. Toosy reported the complete prevention in mice of ulceration seen in all controls by administration of androgens and gonadotropic hormones in males either before or after irradiation by Sr^{90} applicator, with no effect in females. Estrogens protected both sexes when given after irradiation and delayed ulceration when given before. Diethylstilbestrol prevented ulceration when administered after irradiation, but failed to protect when given before irradiation (*57*). Davis *et al.* (*24*), utilizing the agents shown to reduce ionizing radiation damage in whole-body radiation, prevented damage to the skin with nitrite, hypoxia, cysteine, and spleen homogenate. None of these studies have implied or indicated data on carcinogenesis. Shapiro (*38*) in a study of the carcinogenic effect of prolonged exposure to grenz rays reported that the drugs Triquin and chloroquine, which initially appeared to offer some protection to the skin, did not prevent the development of squamous-cell carcinoma.

COMPARATIVE SPECIES EFFECTS

It is of some interest to examine the reaction, arising experimentally or otherwise, of the various animal groups to ionizing radiation. These data exist in extensive detail in terms of X ray and, more recently, electron response expressed as erythema, pigmentation, dry and wet desquamation, and superficial and deep ulceration. There is, however, such potential variation in factors required for uniform comparisons that a practical level is not yet attained. For example, Jacobsen *et al.* (*58*) indicate in their studies of multiple exposures to β-irradiation that the effective dose for wet desquamation is 1460 rad in the depilated rat and 2100 in the clipped rat with an increase of 4 days in latent time. Some authors have combined energy transfer with the anatomic site (*6*, *59*) which represents the only sound basis of comparison.

Many minor species differences may be noted, *e.g.*, human skin forms blisters readily in contrast to the skin of practically all other mammals. The wide distribution of apocrine glands in animals compared to limited distribution in man, existence of cyclic growth pattern of a regional nature, and other morphologic and physiological differences have significance in radiation response. Depigmentation of hair is a well-known animal response not demonstrated in man (*60*). Ellinger (*61*) states that epilation dose for rabbits and guinea pigs is 2 to 3 times that of man. He also states that the dose for permanent epilation is relatively uniform for different animal species, between 2000 and 3000 r. Other data (*59*) indicate that the erythema and pigmentation produced in rabbits at 1000 rep is comparable to that produced in human skin at 1000 and 1180 rep. Because of the known variation in physical factors, one is led somewhat hesitatingly to the biologic statement that skin should not be viewed as species-specific, but rather comparisons must be limited to as precise reactions as possible. Even here there appears to be a difference in early and late responses (*41*).

One special phase of the comparative species data is the discussion frequently encountered regarding the animal that most closely resembles the human from the dermal standpoint. Here the matter of fur produces sharp differences which are not satisfied by clipping or depilation of the animals. Comparability with the rabbit with regard to erythema has been noted (*59*). Certain comparability for burn and radiation response has been indicated for the pig (*6*).

DISCUSSION

An inquiry into the general features of the penetration and absorption of ionizing radiation as they may relate to carcinogenesis of the skin brings out an overwhelming number of facts which do not provide all steps from absorption of radiation energy to malignant change. Response to radiation may be demonstrated experimentally in mouse skin to levels at least as low as 5 r (*62*). Carcinoma arises at sites of recognizable and generally marked prior skin damage, though comparable to the pattern with ultraviolet light (*63*) malignancy may develop without extensive visible local damage. There is an opportunity in the readily accessible skin to characterize the cellular changes, and it is possible that culture and chromosome analysis techniques may add to the impetus that exfoliative techniques initiated.

TABLE 1.—Comparative data on radiation-induced erythema

Skin	Type of radiation	Dose	Reference
Human	100 kvp (X rays)	600 r	*43*
Human	P^{32}	1000–1180 rep	*64*
Rabbit	P^{32}	1000 rep	*59*
Human	200 kv (X rays)	500–600 r	*65*
Human	2 Mev	2000 r	*65*
Human	200 kv (X rays)	550 r	*66*
Human	Radium	1000 r	*66*
Human	Fast neutrons (16 Mev)	110 n	*41*
Human	200 kv (X rays)	650 r	*41*
Human	Sr^{90}	600–1000 rad	*67*
Human	1000 kv (X rays)	1000 r	*68*
Rabbit	100 kv (X rays)	1500 r	*69*
Rat	Sr^{90}	2000–5000 rad	*58*
Rabbit	100 kv (X rays)	2700 r	*70*
Pig	Sr^{90}	1800–2250 reb*	*6*
	Y^{91}	3200–4000 reb*	
	Co^{60}	2000–3000 reb*	

*Dose determined at approximate dermalepidermal junction.

TABLE 2.—Comparative data on radiation-induced tumors of skin

Skin	Type of radiation	Dose	Tumor type	Reference
Mouse	P^{32}	3000 rep	Epidermal carcinoma	*27*
Rat	P^{32}	2500 rep	Epidermal carcinoma	*27*
Rat	Y^{91}	1900–4000 rad	Epidermal carcinoma	*26*
		230–500 rad	Adnexal tumors	
Rabbit	P^{32}	16,000 rad	Fibrosarcoma	*28*
Rat	Electrons	1200 rad	Epithelioma	*71*
Rat	Total-body	660 r	Epidermal carcinoma	*72*
Rat	P^{32}	1–3 μc Intraperitoneally	Epidermal carcinoma	*73*

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ULTRAVIOLET CARCINOGENESIS IN MAN[1,2]

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SUMMARY

The histological and cytologic changes in acute sunburn are described and the relationship to theories of carcinogenesis is discussed, with particular emphasis on damage to the negative feedback systems that control mitotic activity in the basal-cell layer. Some concordance of the different theories can be achieved by the hypothesis that initiation is the production of a somatic mutation and promotion consists of damage to feedback inhibitory mechanisms.—NCI Monogr. No. 10: 407–422, 1963.

THE SUBJECT of induction of cancer by ultraviolet radiation has an extensive literature which has been reviewed by Blum (*1*). I therefore do not propose to engage in another complete review of the subject. Much of the evidence for ultraviolet carcinogenesis in man is based on epidemiologic evidence in which well-melanized skins are nearly immune to the development of these carcinomas, whereas people with white skin exposed to intense solar radiation are subject to the development of skin cancers and even of melanomas. Much of the geographic information on which this interpretation is based is reviewed by other speakers in this symposium. As far as I know there has been no deliberate production of ultraviolet carcinoma in a human being.

Therefore, much of what I have to discuss must be based upon speculation, which is based, in turn, upon indirect evidence. Reporting of my own observation and ideas must also be indirect because my own investigations have been concerned not directly with carcinogenesis but with the study of the acute effect of ultraviolet radiation. These studies have been conducted in the belief that studies of the acute effect may ultimately

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contribute to an understanding of the general problem of carcinogenesis. I shall therefore talk principally about some of the acute changes occurring in human skin when exposed to ultraviolet radiation and try to relate these to various concepts of carcinogenesis. There is a tremendous amount of research activity at present on the primary photochemical events in ultraviolet damage. This is also outside the present discussion.

Blum (*2*) has reviewed the optical factors in skin affecting sunburn and carcinogenesis and also the evidence that both sunburn and carcinogenesis are brought about by ultraviolet radiation shorter than 3200 A (0.32 μ). The carcinogenic wavelengths in sunlight are that small fraction of the total sun's radiation which reaches the earth's surface between 2900 and 3200 A. A brief resumé of the effects of latitude, season, and time of day on transmission of the sunburn rays through the atmosphere has been published recently (*3*).

Tissue absorption of ultraviolet radiation in the range of 2900 to 3200 A requires an aromatic ring structure found either in the purines and pyrimidines of nucleic acids or in tyrosine or tryptophan of proteins.

ACUTE EFFECTS OF ULTRAVIOLET RADIATION ON THE SKIN

After exposure of the skin to ultraviolet radiation there is a variable latent period of up to several hours during which no changes are observed (*4*). Then erythema appears as a manifestation of blood-vessel dilatation. Associated in time and intensity with the onset of erythema (*5*), glycogen is deposited in the basal-cell layer. Other evidence of cellular damage does not appear until approximately 24 hours, when scattered through the upper portion of the malpighian layer are cells with a pyknotic, dense nucleus and a glassy homogeneous cytoplasm which is shrunken around the nucleus, and leaves a clear area outside (*5, 6*). These isolated shrunken cells stain deeply with toluidine blue and Giemsa stain but do not show metachromasia. They do not show the orange fluorescence of ribonucleic acid (RNA) on acridine orange stain. Stained with hematoxylin and eosin they appear to be prickle cells. With Gram's stain the cells show dense staining. With phase-contrast microscopy the cytoplasm appears hyaline without the fibrillar appearance of the dyskeratotic cells in squamous-cell carcinoma and Darier's disease.

The fact that some cells show profound change while the surrounding cells show no change initially is important. The time lag between ultraviolet radiation and the cytologic damage is also of interest. It is well recognized in radiation biology, in general, that changes are produced during the prophase of the mitotic cycle which do not appear until the next mitosis (*7*). The cells in the upper malpighian layer of the epidermis are not undergoing mitosis, but they are undergoing changes leading up to nuclear disappearance in the normal skin. The processes by which the nucleus is broken down and dissipated are not known. However, it would appear likely that at some point the coils of deoxyribonucleic acid

(DNA) have to be "unzipped." If radiation jams this "zipper," as is suggested in ionizing-radiation effects on mitosis, then some type of comparable process may be involved. Some of the latent period of ultraviolet effects might then be interpreted as related to mitotic interval delays.

After the appearance of these individually shrunken cells there is some vacuolization which spreads around several cells. The prickle-cell layer becomes thickened and has increased cellularity. Increase in basal-cell mitoses reaches a maximum 60 to 72 hours after the ultraviolet exposures, or about 48 hours after the evidence of major cytologic damage among the prickle cells. It is of interest that in stripping injury of the skin (*23*, *25*) the peak of mitoses occurs 48 hours after the stripping. The entire upper layer becomes condensed with many heightened staining properties and is then shed with nuclei and condensed cytoplasm. In contrast to the parakeratosis of many dermatologic disorders, the scale which peels from sunburn contains compressed but not flattened nuclei and the cell outlines are not greatly flattened. By 5 days, the formation of a new granular layer is evident underneath the damaged layers which are being removed. In the sections I have studied there is remarkable lack of cellular infiltrate into the damaged epidermis. I have identified only a few lymphocytes and have not detected the migration in mild-to-moderate sunburn of polymorphonuclear leukocytes or of eosinophils. In my opinion, the entire appearance, as sampled by biopsies over a 5-day period, is that the outer portion of the malpighian layer has become hyalinized or concentrated rather than dissolved or broken down. This leads me to believe that the effects of sunburn on the rest of the skin are mediated not by release of toxic materials but by withdrawing mature cells from biochemical activity, particularly the production of organ-specific mitotic inhibitors to the basal-cell layer. Hence, this discussion emphasizes interrupted feedback aspects of carcinogenesis from ultraviolet light in skin. This is undoubtedly only part of the picture. It is well established that ultraviolet radiation produces mutations in germinal cells in insects and in one-celled organisms. It is quite likely, therefore, that it also produces somatic mutations in basal cells of the skin, but there is no way to demonstrate these directly.

One of the experiments which obviously should be done is to produce sunburn in patients who are susceptible to skin cancer and have thin, atrophic skin. It may be, under these circumstances, that cellular changes will be produced in the basal-cell layer rather than among the cells without genetic progeny in the upper malpighian layer.

The latent period of acute ultraviolet effects has long been one of the fascinating features of sunburn. The latent period of the onset of erythema has been explained by the time required for diffusion of a relatively large molecule, the proteose produced by the sunburn damage, in the upper portion of the malpighian layer to reach the capillary.

The chronic effects of repeated ultraviolet exposure in those not adequately protected against it by pigmentation or other skin mechanisms are basophilic degeneration of the connective tissue, fragmentation of the elastic tissue, the so-called picture of the senile elastosis, and carcinogenesis. There has been some dispute whether the senile elastosis represents alteration in elastic fibers or alteration of collagen. Fluorescence microscopy of normal skin suggests the elastic tissue as a suspicious candidate because of the fluorescence of the elastic fibers (*8*). We associate fluorescence with chemical structures capable of activation upon the impact of ultraviolet radiation.

In the future we shall probably have to consider not only macromolecule targets, such as protein and DNA, but also organelle targets for initial and early ultraviolet effects. One subcellular particle which has to be considered when cells undergo sudden destructive processes is the lysosome (*9*). Lysosomes are specific particles, approximately 0.4 μ in diameter, containing a number of hydrolases with optimum function in the acid range of *p*H. Enzymes contained in these particles include acid phosphatase, acid ribonuclease, acid deoxyribonuclease, cathepsin, β-glucuronidase, aryl-sulphatase, and perhaps other enzymes. These structures are ordinarily sealed off, but under certain stimuli they rupture and release these enzymes which are capable of breaking down all the major components of cells. A variety of traumatic stimuli have been described for the rupture of these structures. I have not found reports of ultraviolet in this connection. Novikoff (*10*) implicates one form of the lysosome in keratinization processes and squamous metaplasia. While the release of these enzymes into a cell would more logically lead to lysis of the cytoplasm than to its apparent condensation and homogenization, the lysosome is a structure that must be considered in sudden isolated cell destruction. If the cells in the upper half of the malpighian layer, which show the initial cytologic effect of sunburn, are actually premature dyskeratotic cells, then perhaps lysis of the other constituents has occurred, and keratin is left to form the residual cytoplasmic mass. These considerations are at present speculative and will remain so until these ultraviolet target cells have been adequately studied by ultrastructure techniques.

My personal candidates for light-sensitive organelles are the laminated structures described by Breathnach, Birbeck, and Everall (*11*) in their electron microscope study of the Langerhans' cells of the epidermis. These authors consider Langerhans' cells to be effete nonpigmented cells of the melanocyte series. The structures they describe are reminiscent of other photosensitive structures including the grana of chloroplasts. The distribution of the Langerhans' cells is comparable to the scattered, individually shrunken cells seen in acute sunburn. I am not sure that these cells first involved in sunburn have been completely identified, but my histopathological consultants so far have considered them to be prickle cells. Even though the hypothesis is far from proved, the biological nicety impels me to suggest the possible picture at this time. If the electron-dense and light sandwich with 90 A banding is a light-sensitive structure, then it is conceivable that this is the site of vitamin D synthesis. At least no other known biochemical use of light energy is found in skin except for the photoisomerization of urocanic acid (*12*). We can readily visualize overloading the light-sensitive structure with a heavy dose of ultraviolet radiation. The damage to this cell either releases active mediators, which initiate some of the reactions of sunburn, or a normal feedback regulatory mechanism is interrupted. Since, according to Breathnach and associates, these Langerhans' cells are in excess in basal-cell location in vitiligo, a feedback inhibition of the activity of melanocytes would appear likely. Damage to this feedback mechanism would then be consistent with the upward melanin migration and increased melanization after sunburn.

FUNCTIONS OF MELANIN

In view of the overwhelming epidemiologic evidence from many parts of the world which has been presented in this conference, it may be difficult to understand why there have ever been doubts that melanin in the skin prevents sunburn and cancer formation by absorbing ultraviolet radiation. The first problem has to do with the distribution of melanin in the skin. Examination of a silver-stained section of a normal Negro skin shows that here, as in all other human skin, the melanin is concentrated heavily in the basal layer where it tends to be located as dense supranuclear caps. Melanin is produced in the melanocytes and is apparently injected into the basal cells. Higher up in the Negro skin there are much smaller melanin caps over the nuclei of the prickle-cell layer. If the DNA of the nucleus is the molecule most easily damaged by ultraviolet radiation, as is certainly true for many other types of radiation effects (*7*), this concentration as a supranuclear cap may serve to provide maximum protection with a minimal quantity of melanin. In the Negro skin, and in lighter skins following sunburn, there is a fine scattering of melanin dust in the stratum corneum. These particles show best with a silver stain, are smaller than melanin granules, and quite widely separated. However, these particles of melanin dust are presumed to be the cause of the increased opacity of the Negro epidermis as demonstrated by Thomson (*13*). One of the puzzles about the function of melanin in protecting skin from sunburn has been the observation (*1, 2*) that in spite of the persistence of visible melanization for months or even years the erythema threshold returns to baseline in approximately 2 months in white skin. Miescher (*14, 15*) studied the direct darkening effect of longer-wave ultraviolet and visible light on preformed melanin. He found that the skins in which this reaction occurred had silver-stainable melanin in the prickle-cell layer and the stratum corneum. Presumably, persons failing to show this reaction have melanin almost entirely localized in the basal-cell level.

A further problem is the location of most of the melanin beneath the layers where the primary photochemical reactions occur in sunburn.

Considerations of the depth penetration of ultraviolet radiation and the histologic changes indicate that the prickle-cell layers are the site of the primary damage. One suggestion is that the supranuclear cap provides protection in the basal cells for the genetic material for all the keratinocytes of the epidermis. Since all cells above this level are being modified and shed, as emphasized by Van Scott at this meeting, mutation or other genetic damage produces no long-term harmful effect. The arrangement is then looked upon as a compromise between protection of the genetic material by melanin at a low level and vitamin D synthesis by ultraviolet radiation in the prickle cells.

Other suggestions have been that the melanin acts not only as an optical filter but also by some chemical means, such as reacting with photoactively produced peroxides or free radicals. Melanin has stable free radical properties (*16*). The electron-spin-resonance signal indicating the unpaired electrons of the free radical structure increases upon ultraviolet irradiation (*17*).

An explanation of this general type must be invoked to explain the marked tolerance of heavily pigmented skin to chemical agents (*18*). These explanations are certainly more in line with the claims that heavily pigmented skin is more tolerant of ionizing radiation and chemical carcinogens than is lighter skin (*19*). The optical-filtering properties of melanin can obviously have no effect on these 2 important reactions.

Further complications were introduced by the early investigators (*20*) of ultraviolet radiation who found that within a few hours after exposure the skin became refractory to the additive effect of another dose of ultraviolet radiation. Since changes in melanin pigmentation are not evident before 48 hours, failure to demonstrate additional erythema could not be explained on the basis of melanin pigmentation. It was found that this tolerance to subsequent irradiation was also present in patches of vitiligo and in albinos. The investigators therefore concluded, and they have been extensively quoted (*1*) for many years, that the increased thickness of the stratum corneum, which was evident, must be the filtering factor rather than the melanin. This seems reasonable when tolerance develops before the melanin and disappears before it. These facts are not entirely explained, but it is clear that melanin is not the whole story in the response of the skin to acute sunburn.

In experimental and clinical studies the minimal erythema threshold has been used, a vascular response which may be subject to many other influences. Comparison of the minimal responses has perhaps also been misleading. Miescher (*15*) compared the ratio between the dose that produces minimal observable changes and that which produces permanent damage to the skin as indicated by decrease in pigmentation. He found a ratio of 1 to 8 between a white and Negro skin for the minimal evidence of effect, which was erythema in the white and pigment-darkening in the Negro. A factor of 8 is that described for the effect of doubling the thickness of stratum corneum on erythema threshold according to Rottier (*21*). In the white, the ratio between threshold response and permanent damage was 1 : 64, while in the Negro it was 1 : 960. Some of the apparent discrepancy between the clinical and experimental effects of ultraviolet radiation may grow out of the consideration that the experimental studies are based upon threshold studies which give only a portion of the story. The assumption that the threshold response is highly correlated with the same or different responses at doses far above the energy required to produce the threshold response is probably not valid. This needs to have systematic study, but investigators who have worked with ultraviolet radiation have not found in the white race consistent effects of skin color, hair color, or eye color upon the erythema threshold. This contrasts with the strong clinical impressions and the epidemiologic evidence that chronic actinic damage and skin cancer show important correlations with the degree of pigmentation. In abnormal responses to ultraviolet radiation the threshold of erythema is also often no different from normal subjects.

RELATIONSHIP OF ULTRAVIOLET DAMAGE TO FEEDBACK REGULATION OF EPIDERMAL MITOSES AND TO THEORIES OF CARCINOGENESIS

Blum's extensive studies (*1*, *2*) on the production of ultraviolet carcinoma and sarcomas in albino mice have given him material for extensive mathematical analysis. He has studied the factors of total dose, dose rate, and the time to tumor development. He has pointed out the hazard of the concepts of the "latent period" and "induction period" because of the difficulty in knowing how long a tumor must grow before it becomes visible. Blum concludes that his data indicate that whatever the nature of the process of cancerization, it begins with the first dose of ultraviolet light and continues progressively as long as the doses are given. However, since single doses fail to produce cancer, and in the experiments on white mice Blum found cancer developing only after 50 exposures, the findings were interpreted to indicate that smaller numbers of exposures did not produce tumors within the lifetime of the animals.

While some of us have difficulty in following Blum's mathematical approach, we can recognize the great importance of his finding that carcinogenesis is a progressively increasing phenomenon. The experimental results could be explained in terms of constant acceleration, *i.e.*, the growth rate increased at a constant rate. The findings, therefore, cannot be explained simply as a somatic mutation with a production of a bad little clone. In speculating about a possible mechanism to explain progressive increase in growth rate, Blum advanced a hypothesis that the ultraviolet damage led to progressive accumulation of replicating units of some type which accelerated the cell growth rate.

The evidence that mitotic activity in the basal-cell layer of the skin is controlled by negative feedback inhibition comes from various sources, is consistent throughout the evidence, and, in my opinion, entirely convincing (*22*). The Scotch tape-stripping experiments of Pinkus (*23*) and of Lobitz (*24*) and their co-workers are familiar to dermatologists. When the stratum corneum is removed by Scotch tape as indicated by repeated stripping down to a glistening moist surface, a number of things begin to happen as shown in biopsies. In some cases, within a very few minutes, edema of the basal-cell layer is evident. These changes are followed in a few hours by appearance of glycogen in the basal-cell layer and mitoses which reach their maximum at 48 hours, following removal of the stratum corneum. This in itself is suggestive of interrupting a feedback inhibition of mitotic activity but is possibly also interpretable on the basis of an injury hormone phenomenon. However, it is of interest (*5*, *25*) that when Lobitz produced a transverse middermal cut with a cataract knife there was no burst of mitotic activity in the basal layer. Bullough has demonstrated the same thing and concluded that epidermal injury of some type is necessary to stimulate mitoses in the basal layer (*26*).

Bullough has extensively studied the question whether injury to the skin produces a wound hormone leading to mitotic stimulation, or whether there is normally an inhibitor substance transmitted back from the intact epidermis to inhibit mitoses. Increased mitotic activity for approximately 1 mm adjacent to a cut in mouse skin was consistent with either hypothesis. Using the mouse's ear, Bullough and Laurence (*26*) have clearly shown that an inhibitory substance must be involved. The evidence includes the fact that when a relatively large piece of epidermis is removed from one side of the mouse's ear the maximum mitotic activity on the opposite side of the thin ear is under the center of the defect. A wound hormone effect should be maximum opposite the margins of the epidermal excision.

Weiss and Cavanau (*27*) have developed a model of growth control in mathematical terms and suggest that ribonucleotides are attractive as inhibitors, but state that there is no proof for the role of these particular compounds in feedback regulation of mitotic activity.

Iversen (*28*) has applied these theories of negative feedback regulation of mitotic activity in the basal layer of the epidermis to an analogue computer model and has found satisfactory support by this line of investigation.

Furth (*29*) has emphasized feedback regulatory factors in the pathogenesis of neoplasia, and emphasized that neoplasia can arise from sustained deficiency of restraining force or sustained excess of a stimulating force as host factors. Cytogenic changes can lead to altered responsiveness of the target or regulator.

Druckrey (*30*) has also discussed the role of interrupted negative feedback regulations in carcinogenesis, and has suggested the ways in which cells may be released from the feedback system. Removal of cells from the feedback system, as in tissue culture, or interruption of the centripetal feedback in the organism will lead to continued growth. Normally cells produce organ-specific substances that act as inhibitors to a regulation center which otherwise stimulates the cell. Anything that damages the functional units producing the organ-specific substance is carcinogenic because the cells become progressively unregulated as these units are damaged.

Osgood's concepts (*31, 32*) of growth regulation in normal and malignant cells have grown out of studies of malignancy, growth, and the incorporation of tritium-labeled thymidine in nuclei of tissue cultures of human blood cells. Osgood points out that in the adult there are two kinds of cells. Cells that are capable of unlimited continued mitotic division, he calls alpha cells. There are also derivatives of these cells which are functionally mature and do not have further mitoses. The basal cell of the epidermis is a typical alpha cell and the other cells of the keratinocytes are characteristic of functionally mature nonmitotic cells which Osgood calls n cells. Growth regulation is brought about by inhibition of mitosis in the alpha cells by tissue- or organ-specific labile inhibitors. Any damage which reduces the lifespan of the n cells can lead to additional mitosis of alpha cells to produce new alpha cells. He considers that neoplastic cells are not dedifferentiated but are undifferentiated cells released from the normal tissue inhibitions. Divisions leading to an alpha cell and an n cell are under similar regulations and interruption may be considered to produce hyperplastic reactions. To increase the number of alpha cells an alpha, 2 alpha division is necessary, an alpha, n division leaves the number of alpha cells unchanged. Progressive acceleration in the growth rate of a tumor mass is simply explained by increasing distance required for diffusion of the mitotic inhibitors from the normal mature cells to the dividing alpha cells. Anything which reduces the inhibition of mitoses increases the probability of random somatic mutations.

An area of great ignorance at present is the period intervening between the effects of acute sunburn and the late changes of chronic ultraviolet exposure. These are familiar as thinning of the epidermis, loss of rete ridges, and degenerative changes in the upper connective tissue. It has sometimes been thought that the connective-tissue changes are collagen but also include elastic fibers. The predisposition of such an atrophic skin to cancer formation is more consistent with a decrease in regulatory factors produced by an inadequate supply of normal tissue and cell than it is with an irritation or hyperplasia phenomenon. Loss of regulatory capacity could involve the dermis as well as the epidermis. Several people have suggested that it is the degenerative change in the upper dermis which is the basic lesion in carcinogenesis. However, the work reviewed in this article principally tends to implicate the epidermis. The puzzle of the Oppenheimer phenomenon in which inert materials in sheet form but not powder form in the dermis lead to tumor formation (*33*) is also much more in keeping with the interruption of a feedback inhibitor than with other explanations.

Some of the highlights of Foulds' (*34*) concept of carcinogenesis include the following: He visualizes neoplasia as a dynamic process of development which often advances discontinuously through qualitatively different stages as a result of progression. Progression is considered to be irreversible change in one or more of the characters of neoplastic cells. As a rule, progression occurs in only one of multiple tumors at any given time. The independent progression of tumors is consistent with the observation that only a fraction of the available tissue capable of undergoing progression does so, which indicates that progression depends upon a qualitative change in one part of a tissue and not on a change in environment to which all parts are exposed. In his discussion, Foulds suggests that we get away from the term "precancerous," and, instead, indicate more specifically which cancerous features are shown by any of the earlier changes resulting from carcinogenic stimuli. He also discusses the concepts of Rous that neoplasia involves a process of initiation, and then a a process of promotion which stimulates latent tumor cells to proliferate and form visible tumors. The number and biological characters of tumors produced in these initiation and promotion experiments are determined by the initiating stimulus. The location and time of emergence of the tumors are a function of the promoting agents. Foulds also discusses the 2-stage hypothesis of Berenblum (*35*) in which initiating action produces a sudden irreversible change in the potentiality of a few isolated epidermal cells and, in effect, converts them to dormant tumor cells. Promoting action encourages proliferation and delays the maturation of the dormant cells. One way in which some of these concepts might be combined is that initiation is a somatic mutation and promotion is damage to the mature cells which damages inhibitory feedback.

At this conference we have repeatedly heard that ultraviolet radiation, trauma, and vascular changes are implicated in carcinogenesis. The experiments of Holti are of particular interest in this regard (*36*). He found that vasomotor effects of ultraviolet radiation persisted for as long as 15 months after sunburn. He further found that sunburn plus trauma in the form of pinprick led to the appearance of a persistent telangiectatic vessel, a morphologic change also seen in the precancerous lesion of chronic radiodermatitis.

The possibility that ultraviolet radiation acts as a cocarcinogen is not discussed here. Epstein (*37*) has confirmed other reports that some chemical carcinogens are inactivated by ultraviolet light and that subsequent ultraviolet irradiation offers some protection from the chemical carcinogenic effect. However, the chemicals appear to augment the ultraviolet carcinogenesis. As far as I know, there is no evidence that ultraviolet may act as a cocarcinogen with virus infections. The absence of information does not exclude the possibility.

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DISCUSSION

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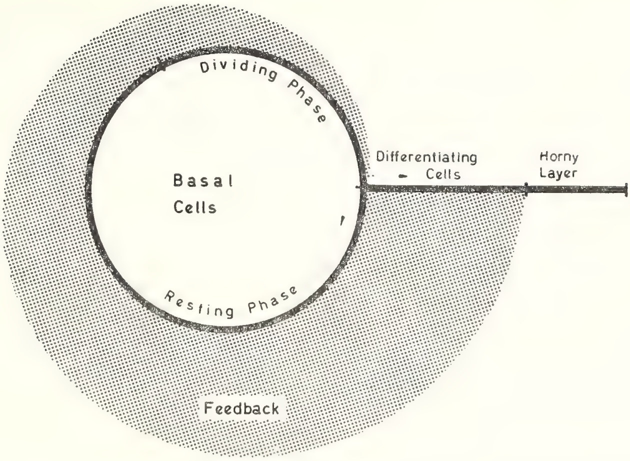
At the Institute for General and Experimental Pathology, University of Oslo, we have been engaged in a study of regulation problems in co-operation with Rolf Bjerknes, research engineer at the Central Institute for Industrial Research, Oslo. We have tried to analyze the growth-controlling system in the epidermis of the mouse and to study the reaction of the epidermis to a single application of carcinogenic substances. The investigation is based on a combination of experimental studies on mice, a construction of a growth model for the epidermis, and the theoretical analysis of this model by means of an electronic analogue computer.

Text-figure 1 shows the essential points in our theory. The basal cells move around the circle, and each time they reach the branching point binary fission takes place. One of the new cells continues as a basal cell, and the other becomes a differentiated cell and ultimately changes into a keratinocyte. The differentiating cells are continuously producing an inhibitory substance which is supposed to diffuse back to the basal cells, as demonstrated, where it controls the dividing rate.

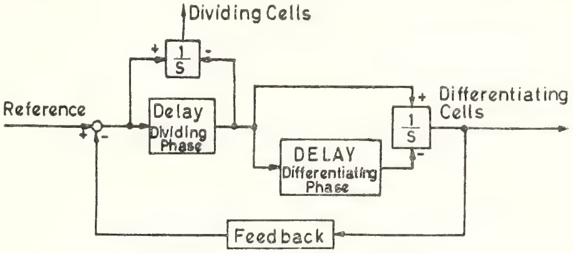
We can speak of a *mean generation time* for the basal cells, which is the most probable time between two subsequent mitoses in the same cell; and we can speak of a *mean turnover time* of the differentiating cells, which is the time from the formation


Dividing Phase
Basal Cells
Differentiating Cells
Horny Layer
Resting Phase
Feedback

TEXT-FIGURE 1.—Deterministic model of life cycle of epidermic cells.

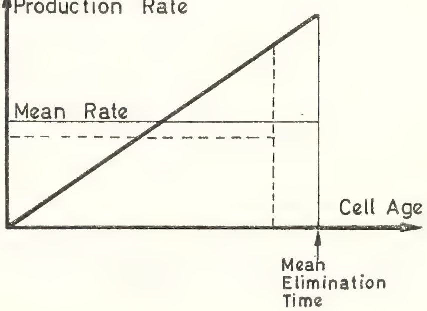
of a new cell until it loses its nucleus and is transformed into keratin. From observations of our mice, we have estimated that the mean generation time of the basal cells is around 5 days, while the turnover time of the differentiating cells is around 3 days.

Text-figure 2 demonstrates a schematic drawing of the model. The two delays, called the "dividing phase" and the "differentiating phase," correspond to the same phases in text-figure 1.


Dividing Cells
+
1/S
-
Reference
+
-
Delay Dividing Phase
+
Differentiating Cells
1/S
-
DELAY Differentiating Phase
Feed back

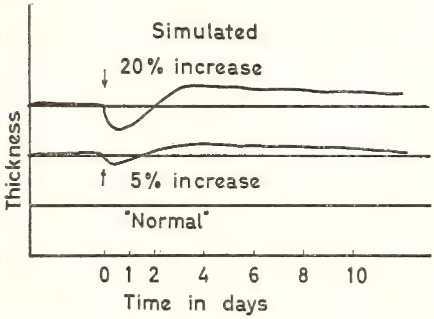
TEXT-FIGURE 2.—Simplified block diagram of model.

To stimulate the feedback production, we must assume a relationship between the production rate and the cell age. A linear relationship, as shown in text-figure 3, seems to be a likely approximation. It follows that the mean production rate is proportional to the mean elimination time. If cells are eliminated at a faster speed than normal (=3 days) the mean level of inhibiting signal will decrease.



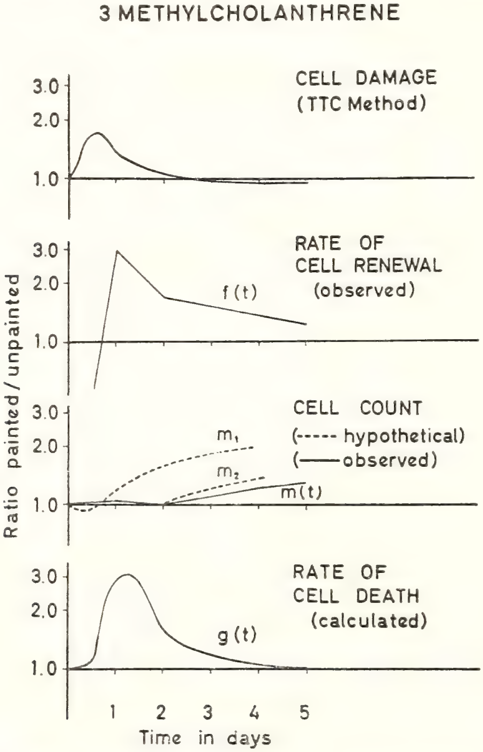
TEXT-FIGURE 3.—Feedback production rate versus age of differentiating cells.

It is stressed that this model is constructed according to our theory and is based on reasoning on biological grounds; no "curve-fitting" is involved. Assuming that this model was isomorphic with the growth-regulating system in the epidermis, we tested the model against a well-known disturbance of the epidermis: increased wear and tear. It is widely recognized that the epidermis reacts to increased elimination rate of horny material by thickening, *i.e.*, the number of cells increases. Text-figure 4 shows the result: The model reacts to increased rate of elimination by increasing the number of cells.



TEXT-FIGURE 4.—Thickness variation of epidermis with increase of rate of cell loss.

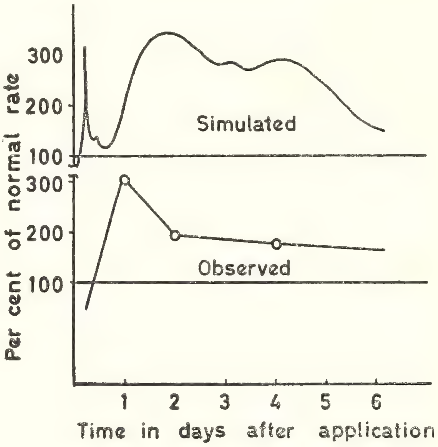
From studies of the effects of a single application of carcinogens to the skin of mice, it has been demonstrated by Iversen and Evensen (*1*) that the following changes in the cell population occur: 1) A very short blocking effect on DNA synthesis and on the



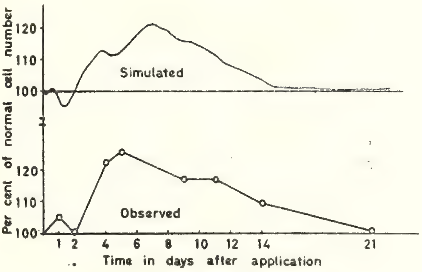
TEXT-FIGURE 5.—Changes induced in epidermal cell population after a single application of 3-methylcholanthrene. Reproduced from Iversen and Evensen, 1962. Oslo University Press, Oslo.

process of mitosis. 2) A comprehensive disturbance of the mitochondria in many cells, followed by the death of many cells. 3) The relatively sudden increase of rate of cell loss is compensated by an increased rate of cell renewal. 4) After 2 days a transient hyperplasia (thickening) develops. Text-figure 5 demonstrates the changes as recorded the first 5 days after the application.

We put disturbances into the model that are aimed to simulate 1) a short blocking of the cell-dividing process and 2) the destruction of mitochondrial function followed by increased elimination of cells. Text-figure 6 shows the simulated rate of cell renewal, which is approximately as observed in the mice. Text-figure 7 demonstrates the change in the number of cells, which is almost identical with the results observed by counting cells in mouse epidermis.



TEXT-FIGURE 6.—Rate of cell renewal after a single application of 1 percent 3-methylcholanthrene.



TEXT-FIGURE 7.—Cell number in epidermis after a single application of 1 percent 3-methylcholanthrene.

Obviously, these results do not tell anything about the development of cancer in mice. But, if the model is based on correct principles, we can conclude that one of the effects of carcinogens may be a destruction of mitochondrial function with subsequent death of the cell. The increased rate of cell renewal and hyperplasia can be explained as a consequence of this destruction. Many theories that assume a "delayed maturation" have been proposed as the explanation of the development of hyperplasia. The results with the model, however, demonstrate that delayed maturation does not seem to be involved; the hyperplasia is a direct consequence of the cell loss.

These theoretical results with the use of a model have given us suggestions as to the planning of new biological experiments.

One of the limitations of analogue computers is that only *quantitative* changes can be tested. It is generally held that cancerization involves a *qualitative* change, initially only in a single cell or in a few cells. Such changes cannot be tested on an analogue computer.

On a digital computer, however, it seems possible to induce qualitative changes in one or in a few units, and to observe the results. We hope to be able to continue the work along these lines.

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X-RAY-INDUCED SKIN CANCER IN MAN[1]

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SUMMARY

Human skin exposed to ionizing radiation may develop squamous-cell and basal-cell carcinoma, occasionally sarcoma, and perhaps rarely, malignant melanoma and sebaceous carcinoma. The dose range required for radiation carcinogenesis in man is not precisely known. While a definite threshold dose cannot be demonstrated, it appears that carcinoma does not commonly develop in the absence of late sequelae. These changes are not usually produced with total doses below 1000 r. The upper limit of the carcinogenic range seems to be below the dose level used for cancer therapy. The mechanism of the genesis of radiation-induced carcinoma appears to be exceedingly complex. However, a considerable body of evidence suggests that perhaps at least two general tissue responses are involved, the direct and indirect,—NCI Monogr. 10:423–432, 1963.

ON OCTOBER 21, 1902, Frieben (*1*) presented before the Physicians Society of Hamburg his well-known case that was perhaps the first recorded instance of cancer resulting from exposure to roentgen rays. This was an epidermoid carcinoma occurring in an area of radiodermatitis on the back of the hand of a worker who used his hand as a test object in a roentgen tube factory. In this earliest period, radiation cancer was an occupational hazard affecting those who worked with radiation apparatus. Soon after, it was reported that cutaneous carcinoma developed in patients who had been treated with the new modality. Thus began the era of "therapeutic radiation cancer"(*2*).

CELL TYPE OF TUMORS OBSERVED

All the early histologic studies indicated roentgen-ray carcinoma was squamous-cell type. These reports evidently strongly influenced subsequent writers who for many years stated that only squamous-cell

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carcinoma developed in chronic radiodermatitis. Textbooks, as recently as 1938, gave the impression that basal-cell carcinoma occurs rarely, if at all, as a result of late radiation changes in the skin. However, in 1951, Anderson and Anderson (*3*) reviewed the historical data regarding the development of basal-cell cancer in radiodermatitis and reported 11 cases of their own. It was their opinion that the cell type of radiation-produced tumors depended on the type and location of the damaged tissue (a view which had been expressed by earlier writers). For instance, one would not expect basal-cell carcinoma to develop in roentgen injuries of the palms and soles where basal-cell carcinoma usually does not occur. The same is true of radiodermatitis involving mucous membranes, such as the lip and buccal mucosa. Likewise, radiodermatitis on the back of the hand would be expected to produce more prickle-cell than basal-cell epitheliomas, because in this area, squamous-cell carcinomas are more common than basal-cell lesions. In 2 recent series of roentgen dermatitis, the preponderance of tumors in the face area was of basal-cell type (*4*, *5*). Allen (*6*) agrees that X radiation can produce basal-cell tumors. We too have found basal-cell carcinoma very frequently in chronic radiodermatitis. The term "carcinoma" is employed here in reference to invasive basal-cell tumors in deference to common usage. However, there is a body of opinion that does not consider these neoplasms true cancers (*7*, *8*).

As early as 1908, Lindenborn (*9*), in a review of the literature on roentgen tumors up to that time, cited 2 cases of sarcoma occurring in areas of radiodermatitis and 3 others in which both carcinomas and sarcoma occurred. While numerous reports of irradiation sarcoma have followed these earlier ones, sarcoma appears to arise in irradiated skin much less frequently than does carcinoma. Indeed, some have expressed doubts that these fibrous proliferations are true sarcomas (*10*).

Stout (*11*) found only 4 small nonmetastasizing fibrous tumors in irradiated tissue (irradiation fibromatosis) in his own material. He questioned whether these should be considered as true fibrosarcomas and preferred to call them "fibrosarcoma-like." Stout conceded that some metastasizing sarcomas have occurred rarely.

Sims and Kirsch (*12*), in 1948, reported 2 cases of spindle-cell epidermoid carcinoma histologically resembling fibrosarcoma occurring in chronic radiodermatitis. In these tumors, the spindle-shaped cells were derived from the prickle-cell layer of the epidermis, but their appearance was so changed that they assumed a fusiform shape resembling connective-tissue cells. The authors considered these morphologic changes due to compression by the sclerosis in the chronic roentgen dermatitis. This appears to be the same type of tumor that Lacassagne (*13*) observed earlier and regarded as an atypical fusiform epithelioma.

It is probable that at least some of the reported sarcomas are in reality spindle-cell carcinomas. Yet it is possible that sarcoma did occur more frequently in these earlier cases that were treated for lupus vulgaris over long periods with X ray. The older German and French literature suggests that sarcoma of soft tissue and bone was more frequent and more malignant if these tissues were the site of chronic inflammatory disease during the irradiation. This observation very early raised the question of the possibility of an additive carcinogenic effect of inflammation.

While undoubtedly of rare occurrence, the development of malignant melanoma has been observed in chronic radiodermatitis. Andrews (*14*) has noted this in 2 instances, and 1 case of malignant melanoma is indicated in the Lacassagne (*13*) series. Likewise, we have observed 1 such case in our own clinical material. Perhaps it is debatable whether these are coincidental, but malignant melanoma occurs occasionally with other tumors in xeroderma pigmentosum, where the histologic changes qualitatively parallel those of chronic radiodermatitis. Also, there is evidence that the incidence of malignant melanoma in light-skinned people may be increased by solar radiation (*15, 16*).

One instance of true sebaceous carcinoma arising in an area of chronic radiodermatitis has been reported by Justi (*17*). This occurred in the skin of the nose of a young man 11 years after the first of a long series of roentgen treatments for a benign skin disease.

RELATION BETWEEN DOSE AND EFFECT

Thus far, it has not been possible to demonstrate in man a precise quantitative relationship between cutaneous cancer development and the amount of roentgen radiation delivered to the skin surface. There are no studies on skin cancer in which a definable population was exposed to amounts of radiation that could be measured or estimated with accuracy. The quantity of radiation absorbed during occupational exposure and that received by patients during diagnostic procedures is usually unknown. Likewise, the dosage given therapeutically before the roentgen unit was used in measurement, that is, before 1930, was quite inexact.

However, there is available some statistical data regarding the dosage of roentgen radiation required to produce definite, visible radiodermatitis. Sulzberger and associates (*18*) examined 1,000 patients who had received superficial roentgen therapy for benign and malignant skin conditions. Single doses varied from 37.5 r for benign dermatoses to 1800 r for cutaneous cancers. The number of treatments per site varied from 1 to 20. These were compared with 1,000 patients who had received no radiation therapy.

From this work, it appears that with the small, fractional doses (suberythema doses) ordinarily used for the treatment of benign skin conditions, a total dose of more than 1000 r was required to produce even faintly visible sequelae. It is of interest that no cutaneous cancer had yet developed in any of these patients, including the 28 who had sequelae and who had received doses higher than 1000 r. Twenty-five of the latter had received cancericidal doses for malignant skin conditions. Miescher and Böhm (*19*) found no late radiation sequelae in patients who had received total doses of 1400 to 1800 r for hyperhydrosis. While these figures suggest a sort of minimum dosage range of roentgen rays required to produce mild roentgen dermatitis and the potentiality of subsequent carcinoma, further quantitative data are lacking.

It is not known which total dose, increment doses, or intervals between the increments would produce the maximum number of carcinomas in human skin. Nor is it known precisely at beyond what dose cancers are no longer produced.

On the other hand, empiric observation provides us with some approximate estimates regarding some of these questions. Radiotherapists have long known that carcinomas occur most profusely in roentgen dermatitis that has been produced by the total of many small doses administered at intervals over a considerable period. Rarely is a new carcinoma seen in the radiation sequelae produced by large doses of roentgen rays administered for the treatment of cancer. This is usually a total dose of 4000 to 6000 r, given within 1 to 4 weeks. In over 6,000 cases of primary internal cancer such as cervix, larynx, and others treated through skin fields, Murphy (*20*) has never seen a skin carcinoma develop in the sequelae.

Quantitative data derived from animal experimentation on radiation carcinogenesis obviously has limitations, so far as its direct application to man is concerned. It is only since the use of the electron beam and β-rays from radioactive emitters that it has been possible to limit the action of radiation to the skin in small animals without injuring the internal organs. While much of this data have not been very rewarding in approaching the problem of establishing a precise dose range of the carcinogenic effects of roentgen rays in human skin, there are some animal responses which suggest a parallel to experience in humans. Henshaw *et al.* (*21*) call attention to one of their experiments in which a group of rats receiving a single dose of 4000 to 5000 rep of β-rays experienced a greater tumor response (more and larger tumors) than a comparative group that had received a single dose of 8500 rep. To these investigators, this suggests an optimal dosage below 8500 rep for tumor induction. Mottram (*22*) in producing cutaneous tumors in mice with the combination of β-radiation and a chemical carcinogen found that no tumors were produced with maximum dosage where the epidermis was destroyed. Much material has been published concerning the effect of various fractionations of dose and comparisons with single total doses. However, the conflicting results are difficult to interpret.

As yet, animal experimentation has failed to demonstrate clearly any definite threshold dose for carcinogenic effect. Mole (*23*) is of the opinion that the presence or absence of a threshold can never be empirically proved, because there is always the possibility that observation on a much larger scale would show tumor responses greater than the control.

It is not surprising but worth noting that late sequelae and a subsequent squamous-cell carcinoma have been observed in man as a result of large total doses of very soft roentgen rays (grenz rays) (*24*). Also, Shapiro *et al.* (*25*) produced squamous-cell carcinomas in mice with 30,000 r of grenz rays, but not with 20,000 r.

Tessmer and Brown (*26*) reported squamous-cell carcinoma occurring in areas of radiation-damaged skin in a cow exposed to the β-radiation of radioactive fallout. This occurred 15 years after inadvertent exposure, following the detonation of a nuclear device near Alamogordo, New Mexico.

CARCINOGENESIS

Shortly after the first observations of radiation-induced cancer, theoretical concepts of carcinogenesis were formulated in an attempt to explain the new phenomenon. Some of the earliest writings on the subject were surprisingly profound and detailed.

In 1904, Unna (*27*) described clearly and minutely the histologic findings in 4 cases of late radiodermatitis. He also pointed out the similarity of these changes to those seen in senile skin and xeroderma pigmentosum. Wyss (*28*) and other early observers stressed the destruction of the corium in the cases of so-called X-ray carcinoma as a confirmation of Ribbert's theory (*29*) that the cellular changes in the connective tissue were the most important factors in the development of all cancer.

Wolbach reported his extensive studies in several communications (*30, 31*). He concluded that the factors responsible for the acquisition of proliferative and eventually malignant properties are changes in the blood supply and connective tissue. He believed that the epidermis is not injured by the roentgen rays passing through, but becomes altered because of progressive impairment of nutrition from a cicatrized corium. Keratinazation is interrupted and the cells remain attached to one another, which results in thick accumulations, thereby forming keratoses. The attempt at maintenance of the continuity of the epidermis initiates a mechanism whereby constant and ultimately accelerated proliferation is called forth. This increase in the power of growth ultimately is accompanied by a loss of differentiation of the epithelial cells that finally become able to derive their sustenance at the expense of the other living tissues.

From the very earliest cases encountered, it was evident that there was a delay in the appearance of the late cutaneous effects of radiation. In the case material assembled by Glucksmann (*32*), the latent period for the development of skin cancer varied from 3 to 48 years. Such a relatively long delay in the development of malignancy has suggested the possibility of an "indirect" (extracellular) effect as a carcinogenic mechanism induced by radiation.

Several statistical studies have been done to determine the incidence of malignant change in patients with so-called radiodermatitis. In the 2 largest series, the figures vary from 10 percent in the study of Saunders and Montgomery (*33*) to 28 percent in the material of Teloh *et al.* (*34*). It is quite probable that the figures would be considerably higher if the patients had been observed over a longer period, and the rates calculated by the life-table technique.

In the same material, at the time of examination, the cases with mild radiodermatitis presented malignant change in less than 1 percent, while in the group with severe damage, 30 percent showed malignant lesions. This suggests some correlation between degree of tissue injury and malignant change in human skin that has received large total doses of roentgen rays in small increments over a long period.

Thus far, an enormous mass of data on the mechanism of radiation carcinogenesis has been accumulated. Recent developments have been reviewed in detail by Brues (*35*) and Glucksmann (*32*, *36*).

For years, there has been speculation as to whether radiation damage to the epidermis, the vascular network, or the connective-tissue elements of the corium is the most important factor in the production of cutaneous cancer. More recently, the significance of the connective-tissue responses to various carcinogens has been again receiving more attention.

Gilman *et al.* (*37*) point out that all skin cancer occurs in areas of degenerated collagen and that perhaps disturbances of the connective tissue alone may bring about hyperplasia and invasiveness of the epithelium. In their elaborate and detailed experiments, connective tissue rendered elastotically degenerated was applied to a Tiersch graft donor site. The regenerating epithelium then became invasive and resembled new growth. They believe these changes are identical in quality, if not in quantity, with those seen in human skin as a result of aging and radiation effect. These workers further speculate on the possibility of producing actual cancer if one were to keep repeating the application of the abnormal connective tissue to the donor site. This mechanism simulates a carcinogen, altering the connective tissue which cannot respond to the epidermal downgrowth in its attempt to form hair papillae. Thus, the epithelial proliferation becomes uncontrolled, *i.e.*, neoplastic. They conclude that perhaps the dermis plays a dominant role in determining whether or not cancer will develop in regenerating epidermis.

This concept appears to have further support in the work of Mackie and McGovern (*38*) who studied the role of collagen degeneration as a mechanism of solar carcinogenesis. In their very detailed histologic and clinical observations, they concluded that collagen degeneration by altering the nutrition of the epidermis predisposes to the development of solar keratosis, keratoacanthoma, squamous-cell carcinoma, and basal-cell carcinoma, and that when collagen degeneration is present in only a moderate degree, it can intensify other carcinogenic influences. It is relative to our present discussion that the connective-tissue changes, which they observed in their cases due to the absorption of ultraviolet light in the dermis, was scarcely distinguishable from chronic roentgen dermatitis, just as Unna had noticed over half a century earlier. Similar alterations in the corium occur in association with skin cancer induced experimentally by methylcholanthrene.

Glucksmann (*36*) regards radiation as a carcinogen that creates environmental conditions for malignant change in regenerating cells rather than directly inducing malignant change in the irradiated cells. One might think of dependent neoplasia as a sort of fantastically exaggerated defense mechanism that has gone out of control. Bryan (*39*), in a recent review, considers neoplasia as a type of tissue response to injury. As the stimulus is prolonged, the proliferation continues beyond the limits of repair and gradually evolves into dependent neoplasia. For such cellular responses that can be compared to inflammation, but which go beyond normal physiological limits, he suggests the term cytoplasia. This concept of cancer developing as a result of attempt at repair is reminiscent of Wolbach's position in 1909.

Yet, in spite of the impressive evidence, the role of connective-tissue damage in indirect carcinogenesis must not be accepted to the exclusion of other mechanisms. Indeed, as Stewart (*40*) points out, systemic diseases which exhibit fibrinoid degeneration of collagen such as lupus erythematosus, rheumatic fever, and periateritis nodosa, do not appear to be related to cancer.

Quite understandably, the question of direct carcinogenic effect of radiation on the epithelial cells has also received considerable attention. Teloh *et al.* (*34*), as a result of their histologic studies, reject the importance of the connective tissue and vascular changes and conclude that carcinoma is produced through a direct effect on the epidermal portion of the skin. In their material, areas of dysplasia were frequently not associated with excessive epithelial proliferation, telangiectasis, or thrombosis of capillaries. The proliferating epithelial downgrowths, regarded by Wolbach as precursors of neoplasia, were merely incidental, because they were also observed in contiguous nondamaged areas.

The dysplasia or dyskeratosis of epidermal cells manifested by hyperchromatic and monstrous nuclei, clumping of chromatin, increased and abnormal mitotic activity is generally regarded as a precancerous change. The precise physiopathologic mechanism whereby a carcinogen produces this effect is not clear. However, it is known that in ionizing radiation the radiant energy on impact is absorbed by the molecules, some of which are ionized (electrons ejected) and some excited (electrons shifted in orbit). Beyond these physical processes during the latent period up until the physiologic or morphologic end-effect, such as the breaking of a chromosome, or the mutation of a gene, our knowledge is fragmentary (*41*).

It appears that enzymes involved in cellular respiration, particularly those containing sulfhydryl compounds, may be altered by irradiation. Also, anomalies in cell division and mutations perhaps may be explained by the inactivation of the enzymes catalyzing the synthesis of new molecules of nucleoproteins. The formation of hydrogen peroxide in irradiated aqueous solutions suggests that this substance, if formed as a result of ionization of intra- or extracellular water, may be toxic to cell constituents. It is quite likely that some of these reactions, and many more unknown ones, occur in a living irradiated cell, but as yet we cannot be certain that any one or a combination of several is responsible for a specific biologic end-effect. Inquiry in this direction leads naturally into the broader subject of the nature of all neoplastic phenomena. A comprehensive critique of this problem as it is approached by diverse disciplines has been published by Furth (*42*).

In the production of radiation-induced skin cancer, as in other neoplasms, the possibility of the existence of cocarcinogenic factors must be considered. Mottram (*22*) demonstrated in mice that, under the conditions of their experiments, subcarcinogenic doses of β-radiation and benzpyrene when combined produce benign and malignant epithelial tumors, but with a shorter latent period than when the chemical alone is applied in carcinogenic doses. Similar additions of radiation and chemical carcinogens have been employed by others.

The extensive work of Berenblum (*43*) has contributed much supporting evidence for the 2-stage-mechanism hypothesis of carcinogenesis. This postulates an initiating phase in which normal cells are transformed into dormant tumor cells, followed by a promoting phase in which these dormant cells are in some way stimulated to produce a visible tumor. For example, in the skin of mice urethan was applied as an "initiator" and subsequently croton oil applications were used as a "promoter" resulting in visible tumors. It is conceivable that natural systemic initiators also exist. The studies of Ghadially (*44*), Quevedo (*45*), and others suggest that the cycle of hair growth may influence the action of cutaneous carcinogenesis. While none of these mechanisms has, as yet, been implicated in cutaneous radiation carcinogenesis in man, the possibility that somehow they may influence our concepts of latent period and "premalignant" cannot be dismissed.

We might conclude that both direct and indirect mechanisms operate in the production of radiation-induced cancer of the human skin. Further complexity is suggested by the possibility that in addition to radiation, other factors, as yet undefined, may exert a synergistic or cocarcinogenic effect.

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VIRUSES AND SKIN TUMORS IN MAN AND ANIMALS[1]

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SUMMARY

Intensive research in the past 10 years has implicated viruses as the cause of many animal tumors, and it is probable that some malignant human tumors may be caused by viruses. In this presentation, the skin tumors known to be caused by viruses are discussed, and some aspects of tumor immunity and possible mechanisms of virus-induced tumors for motion are presented.—NCI Monogr. No. 10: 433–445, 1963.

TEN YEARS ago the suggestion that viruses might cause human malignancies was not considered seriously in scientific circles. Today opinion has changed. Intensive research has implicated viruses as the cause of many types of tumors in laboratory animals. Such animal tumors as malignant carcinoma of the breast and kidney, sarcoma, leukemias, lymphomatosis, and erythroblastosis have been shown to be of viral origin. It seems probable, from what has been learned from animal experiments, that some malignant human tumors are caused by viruses, although this has not yet been established.

For 50 years after the demonstration of tumor viruses by Rous, the fowl tumors were considered by many to be biological freaks with no relationship to tumors in mammals or man. Now most cancer workers agree with Osler's remark that "there is only one medicine." Biological processes in one species, order, or phylum are found in slightly altered form in another. The variations at the cellular level are minute, complex, and seemingly endless, but the basic patterns are repeated.

In this presentation, I will adhere to the subject assigned and discuss mainly skin tumors caused by viruses. The best definition of a virus that fits all the known facts is the concept advanced by Luria (*1*) from studies with *Eschericha coli T* phages: ". . . a genetic element of the cell with specialized ability to produce a protein jacket for itself to facilitate transfer to other cells or other hosts."

Studies with bacteriophage have resulted in the concept that a virus loses its identity upon entering a cell and a new system is formed which is not simply a host cell containing a parasitic virus, but a cell-virus complex. The virus may become completely integrated into the cell with no detectable change, or it may alter and rechannel the energy of the cell in a number of ways. The following results can be observed in various host-virus systems:

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1) The virus may remain inactive and pass to each daughter cell at mitosis, but be recoverable following suitable stimulus, as in lysogenic T phage.

2) It may lose its identity but alter specific cell products as in *Corynebacterium diphtheriae.*

3) It may divert the host cell to production of virus particles at the expense of host-cell elements, with eventual lysis of the cell and liberation of many new virus particles, as in polio viruses.

4) It may cause production of new virus particles without lysis of the infected cells, leading to accumulation of a mass of abnormal cells, filled with virus, as in molluscum contagiosum.

5) It may stimulate growth and excessive multiplication of host cells as in warts and papillomas.

6) It may stimulate the cell to proliferative activity, the end product of which is neither more virus particles nor more host cells, but a new cell which has new and different characteristics. This new cell may be unable to compete with normal cells, and be destroyed; or, it may be more aggressive and produce benign or malignant tumors.

(*a*) Some virus may be produced along with the new cell type, as in Rous sarcoma or adenocarcinoma of the frog.

(*b*) The virus may disappear after causing or setting the stage for an anaplastic change in which the virus is no longer needed to enable the cell to escape the normal regulatory mechanism—as in carcinoma following Shope papilloma.

7) It may produce results which are combinations of the effects listed, or a situation in which different effects predominate at various times in the life cycle of the host or in response to environmental or endocrine changes.

At least 36 tumors known to be caused by viruses are listed in tables 1 and 2. More than half of these are tumors of the skin and appendages, chiefly benign papillomas, or fibromas. The only human tumors known to be caused by viruses fall in this group of warts and papillomas.

Table 1 shows, in chronological order of discovery, 17 tumors caused by viruses in 7 laboratory animal species. They include 3 major tumor groups: leukoses, or tumors of the blood-forming elements; sarcomas, tumors of connective tissue; and carcinomatous tumors of epithelial origin. The animals involved are amphibian, avian, and mammalian species.

In 1908 Ellerman and Bang (*2*) successfully used filtrates to transmit erythromyeloblastic form of chicken leukemia, and in 1911 Rous transmitted the first solid tumor, Rous sarcoma, by a filtrate (*3*).

In spite of this work, the virus theory of cancer was not widely accepted, partly because virologists and virus techniques were not available, and

TABLE 1.—Mesenchymal tumors caused by viruses

Year of discovery	Type of tumor	Host	Investigator
1908	Erythromyeloblastosis	Chicken	Ellerman and Bang (*2*)
1911	Sarcoma	Chicken	Rous (*3*)
1936	Mammary tumor	Mouse	Bittner (*46*)
1938	Kidney carcinoma	Frog	Lucké (*47*)
1946	Lymphomatosis	Chicken	Burmester (*48*)
	Osteopetrosis	Chicken	Burmester (*48*)
1951	Leukemia	Mouse	Gross (*49*)
1956	Chloroleukemia	Mouse	Graffi (*50*)
1957	Reticulum-cell leukemia	Mouse	Friend (*51*)
1957	Polyoma (sarcoma and carcinoma)	Mouse	Stewart and Eddy (*28*)
	Sarcoma	Hamster	Eddy and Stewart (*29*)
	Sarcoma	Rat	Eddy and Stewart (*30*)
	Sarcoma	Guinea pig	Eddy (*31*)
	Fibroma	Rabbit	Eddy and Stewart (*32*)
1960	Leukemia	Mouse	Moloney (*52*)
1961	Sarcoma-SV-40	Hamster	Eddy (*34*)
1962	Sarcoma-adenovirus 12	Hamster	Trentin (*36*)

partly because other etiologic factors were discovered. Thus, in 1918, Yamagiwa and Ishikawa (*4*) produced carcinoma in rabbits by repeated application of tar to the skin. In 1929 Richter and MacDowell (*5*) demonstrated the importance of genetic factors in the genesis of mouse leukemia. In 1936 Furth and Furth (*6*) showed that exposure to X rays may produce leukemia in mice. In some cases, such as mammary cancer of mice, tumors were shown to result from the interaction of three factors acting together: genetic background, hormones, and a virus (*7*).

Further recent studies in this field, however, have shown that the tumorigenic effect of nonviral carcinogenic agents do not definitively rule out viruses as a cause. It was shown, for example, that chemical carcinogens may activate or potentiate the effectiveness of viruses with respect to carcinogenesis. In any event, the number of malignancies proved to be of viral origin has become too large to be ignored.

Table 2 shows 14 skin tumors caused by viruses in 9 mammalian species, including man. These are of sufficient interest to discuss in some detail.

TABLE 2.—Skin tumors caused by viruses

Year of discovery	Type of tumor	Host	Investigator
1898	Myxomatosis	Rabbit	Sanarelli (*8*)
1905	Molluscum contagiosum	Human	Juliusberg (*9*)
1919	Warts	Human	Wile and Kingery (*12*)
1920	Warts	Cattle	Magalhaes (*13*)
1923	Laryngeal papilloma	Human	Ullmann (*45*)
1932	Oral papilloma	Dog	DeMonbreun (*14*)
1932	Fibroma	Rabbit	Shope (*20*)
1932	Papilloma	Rabbit	Shope (*17*)
1936	Oral papilloma	Rabbit	Parsons and Kidd (*16*)
1951	Papilloma	Horse	Cook and Olsen (*15*)
1953	Fibroma	Gray squirrel	Kilham (*23*)
1955	Infectious fibroma	Deer	Shope (*26*)
1957	Polyoma	Mouse	Stewart and Eddy (*28*)
1958	Histiocytoma	Monkey	Bearcroft and Andrews (*53*)

MYXOMATOSIS

In 1898 Sanarelli transmitted rabbit myxomatosis by filtrates (*8*). This is a highly contagious, rapidly fatal disease characterized by blepharo-conjunctivitis and firm, tumorous nodules in the skin around the nose, mouth, ears, and genital organs, leading to death within 2 weeks. Upon histologic examination, the tumors resemble gelatinous myxomatous tumors in man. The disease is indigenous to Brazil and Uruguay where wild rabbits develop only benign fibromatous tumors. The rabbit is the only susceptible animal.

This virus-induced tumor has had an interesting subsequent history. It was introduced artificially into Australia in an attempt to reduce the population of wild rabbits, which are descendants of European domestic rabbits. In Australia, the disease is transmitted by the mosquito, *Anopheles annulipes*. In the wet seasons of 1951 and 1952, a large number of rabbits succumbed to the disease.

MOLLUSCUM CONTAGIOSUM

Early studies by Juliusberg (*9*) established the causative agent of this tumor as a filterable virus, the largest member of the pox group of viruses. It is readily transmitted to man after inoculation into the skin. The incubation period is 14 to 50 days, followed by a papule which enlarges up to 10 mm in diameter, producing a bulbous, pearly, raised nodule which is indented at the top. These nodules persist for weeks to months and regress if subjected to trauma or if they become secondarily infected (*10*).

The tumor consists of a large mass of closely packed epithelial cells pushing into the corium without breaking the basement membrane and extending above the surrounding skin. Cells near the base of the papule are grossly normal, but as they progress toward the surface, the cytoplasm becomes progressively more distended with deoxyribonucleic acid-(DNA) staining material separated by thin strands of cytoplasmic material. Near the surface, dense masses fill the huge cells, flattening the nucleus to the periphery of the cell. By proper techniques, the large cytoplasmic masses are shown to be composed of virus elementary bodies. Recurrence is rare.

WARTS

Ciuffo in 1907 (*11*) and Wile and Kingery in 1919 (*12*) transmitted warts from man to man by intradermal inoculation of filtrates. This has been repeatedly confirmed. The incubation period is 3 to 6 weeks and one virus appears to cause different wart types, depending on the site of inoculation, *i.e.*, flat warts (verrucae planae), palmar and plantar warts, common warts (verrucae vulgares), filiform warts, moist warts (condylomata accuminata), and laryngeal papilloma.

Warts are most common in school children and young adults. They may persist for a few weeks or for years, but frequently regress after 2 to 3 years. Recurrences are not rare. Warts rarely become malignant, though this has been observed in moist, genital warts. Therapy must be individualized, but psychotherapy, roentgen ray, podophyllin, electrodesiccation, and chemical destruction are most successful.

The basal-cell layer shows evidence of rapid proliferation and there is great thickening of the granular and keratinized layers. Long, thin papillae extend high into the wart and may bleed if the wart is trimmed. Dense DNA-staining intranuclear inclusions are seen in the thickened prickle-cell layer, and cytoplasmic inclusions have also been described (*10*).

Warts in other animals are similar in most respects to warts and papilloma in man. In cattle, they are most frequently seen on the face, neck, and shoulders of young animals and also on the udders of adult cows (*13*). They tend to disappear as the animals age, but may persist and become pedunculated.

PAPILLOMATOSIS

Dog.—Oral papillomatosis in dogs (*14*) occurs sporadically or as epizootics chiefly among puppies. The incubation period is 4 to 6 weeks, and papillomas tend to disappear in about 6 weeks. The virus produces papilloma only when inoculated into the mucous membrane of the mouth, and only the dog is susceptible. Recovery is followed by resistance to reinfection.

Horse.—Cutaneous papillomatosis on the lips and nose of the horse (*15*) was observed by Cook and Olsen. The papillomas could be passed with filtrates to the skin of the neck but not to other animals. Tumors regressed spontaneously and the animals were resistant to reinoculation.

Rabbit.—The virus of oral papillomatosis of rabbits produces tumors only in domestic and wild cottontail rabbits, the most successful site being the undersurface of the tongue. No lesions are produced when infectious filtrates are inoculated into the mucous membranes of the nose, eye, conjunctiva, or genital organs (*16*). Following inoculation under the tongue, papillomatosis growths appear within 2 to 4 weeks, persist for several months, and then regress. These rabbits are immune to reinoculation. The virus is sometimes found in the saliva of normal rabbits. There is no cross-immunity with the Shope rabbit papilloma virus.

Monkey.—Monkey histiocytoma or Yaba virus is discussed in the following article by Dr. Ambrus.

Rabbit.—Rabbit papilloma described by Shope in 1933 (*17*) is one of the earliest and most intensively studied mammalian virus tumors. Like the other papilloma viruses it is filterable and highly specific for the skin of the rabbit—causing no papillomas if injected into any other tissue or organ. It produces no lesions in other laboratory animals.

In the wild cottontail rabbit, large, horny papillomas are produced that contain abundant infectious virus in the upper layers, while in the domestic rabbit, papillomas are produced which contain immature, noninfectious virus, which cannot be demonstrated by electron microscopy. The noninfective virus retains its antigenic potency and can be used to immunize rabbits against infective virus. Syverton (*18*) showed that 25 percent of wild rabbits and 75 percent of domestic rabbits kept for more than 6 months developed carcinoma at the base of the papilloma. These carcinomas metastasized and killed the host. No papilloma virus was found in metastatic carcinoma from either the domestic or cottontail rabbits and viral antigens could not be demonstrated immunologically. Later studies (*19*) have shown that some strains of carcinoma contain viral antigen while others do not.

The histologic appearance of the Shope papilloma is similar to other viral papillomas. The carcinoma developing from it is a squamous-cell carcinoma.

Wild and domestic rabbits can be immunized against papillomatosis by parenteral injection of infectious or noninfectious papilloma suspension, and after an interval the blood serum contains neutralizing antibodies.

FIBROMAS

Rabbit.—Fibromas were observed under the skin of the feet of wild rabbits by Shope in 1932 (*20*). These were transmitted to wild and domestic rabbits with a filtrate injected into or beneath the skin. The fibromas grew for a few weeks, then remained stationary and eventually regressed. They were not contagious by contact, but could be transmitted by biting insects.

Rabbit fibroma virus is related to rabbit myxoma virus as shown by cross-resistance to reinfection. Furthermore, myxoma virus inactivated by heating to 75° C, when added to live fibroma virus and administered to domestic rabbits, produces fatal myxomatosis. This phenomenon of recombination indicates the close relationship of the two viruses (*21*, *22*).

Gray squirrel.—Fibromas of the skin of gray squirrels were transmitted to suckling squirrels with filtrates by Kilham in 1953 (*23*). Fibromas developed in 2 weeks and regressed after a period of weeks in most animals. In a few animals, metastatic tumors developed in the skin and lungs.

Young woodchucks also developed skin tumors after injection of squirrel fibroma filtrates. The virus could be transmitted from one squirrel to another by mosquitoes.

The squirrel fibroma virus is closely related to the Shope rabbit fibroma virus, as shown by histology of the tumor and by the presence of cytoplasmic acidophilic inclusion bodies. The squirrel fibroma virus is completely neutralized by Shope rabbit fibroma-immune serum. Shope rabbit fibroma virus does not induce tumors when inoculated into squirrels, but squirrel fibroma virus induces tumors when injected into rabbits (*24*). Furthermore, recombination could be demonstrated when heat-inactivated myxomatosis virus was mixed with squirrel fibroma virus.

Multiple pulmonary adenomatosis was produced (*25*) in young squirrels by intratracheal inoculation of squirrel fibroma virus. These tumors were composed of papillary bronchial epithelium containing eosinophilic inclusion bodies.

Deer.—Infectious fibromas of deer, transmissible by filtrates, were described by Shope in 1955 (*26*). Multiple tumors on the head, neck, and sides varied from minute to 10 cm in diameter and were flat to pedunculated. After injection of Berkfield N filtrates into the skin of young deer, small, multiple fibroma appeared at the site of injection in 7 to 8 weeks.

POLYOMA VIRUS TUMORS

Polyoma virus, first described by Gross as parotid-tumor virus (*27*), was isolated by passage in monkey kidney tissue culture by Stewart and Eddy *et al.* in 1957 (*28*). This is a most exciting tumor virus because of its many striking properties and the stimulus it has given to virus tumor research. Some of the interesting facts about polyoma virus may be mentioned briefly:

Parotid-tumor extracts contain relatively little virus as shown by tumor production when filtrates are injected into newborn mice. These tumors are mostly parotid and, less often, subcutaneous sarcomas. However, when parotid-tumor extracts are passed in tissue culture, a marked enhancement of virus titer and tumorigenicity is achieved. Over 90 percent of Swiss mice, given injections at 1 day of age, develop parotid tumors by age 3 to 4 months. Over 40 percent also develop thymic epithelial tumors. About 30 percent have mammary carcinomas and 20 percent renal sarcomas. Other mice develop osteogenic sarcoma, multiple benign epithelial tumors of skin appendages, buccal mucosa carcinoma, liver hemangiomas, and adrenal medullary tumors. Twenty-three different tumors have been described (*24*).

The polyoma virus produces a cytopathic effect in mouse-embryo tissue culture and can be titrated in a manner similar to other cytotoxic viruses. Virus titers of 10^{-7} are achieved in mouse-embryo tissue culture. It also causes agglutination of red blood cells of man and many laboratory animals. Thus, rapid and sensitive laboratory tests are available for quantitative titration of the virus.

Tumor production by polyoma virus is not restricted to one species. Tumor induction is faster in hamsters than in mice (*29*). Two to 3 weeks after day-old Syrian hamsters are inoculated, sarcomas develop in the kidneys, intestines, heart muscle, and subcutaneous tissue. Cavernous hemangiomas are common in the livers of such animals. Day-old rats inoculated with polyoma virus develop sarcomas of the kidney and subcutaneous tissue plus occasional cavernous hemangiomas of the liver (*30*). Sarcomas are induced in the guinea pig (*31*) and fibromas are induced in the rabbit (*32*) if the animals are inoculated within 24 hours after birth. No polyoma virus antibody has been found in domestic large animals, in wild animals except mice, or in cancer patients.

Polyoma virus is a good antigen and induces specific antibodies when injected into rabbits, guinea pigs, or mice. Antibody can be demonstrated by complement fixation, virus neutralization, tumor neutralization, or hemagglutination-inhibition tests. It is not antigenically related to any other known virus. The virus is fairly heat resistant and requires 70° C for 30 minutes for inactivation. It is stable at −70° C, in 50 percent glycerin, or on lyophilization. It resists 2 percent phenol and 50 percent ethyl alcohol.

Polyoma virus is widely distributed among mouse colonies. In infected colonies, mothers transmit protective antibodies to their young via the placenta and milk. The young mice, when weaned, lose their antibodies and gradually become infected by inhalation or ingestion. Infection at this age leads to a low incidence of tumor formation in contrast to the high rate observed when newborn mice are artificially infected with large doses of virus. In a naturally infected colony, most old mice have specific antibody in their serum while most young mice do not. In such a colony, tumor induction is uncommon to rare, parotid tumor in older mice being the most frequent manifestation. Thus polyoma virus in mice is transmitted horizontally (by contact). In contrast, another well-studied tumor virus—avian lymphomatosis—is transmitted vertically from one generation to the next through the egg, even though the mother, the egg, and the chick contain measurable neutralizing antibody. When injected into the newborn mice, polyoma virus multiplies in all tissues of the body. Greatest virus concentration is found in the salivary gland and kidney. After 2 months, virus multiplication falls to low levels and tumors first appear, reaching an incidence of 50 percent only after 4 months. In newborn Syrian hamsters, the same sequence is observed, but the time is shortened to 3 weeks.

In summary, polyoma virus does not exhibit many of the distinguishing characteristics proposed by Furth for tumor viruses, *i.e.*, species specificity, strain specificity, cell specificity, and low antigenic activity (*33*). In these and many other aspects, it behaves more like ordinary viruses. Since a cancer virus such as polyoma can behave like an ordinary virus, it has been speculated that other common viruses may, under certain circumstances, induce tumor formation. The most dramatic support of this hypothesis comes from the demonstration by Eddy that an adenovirus of monkeys, SV-40, causes subcutaneous sarcomas in 100 percent of newborn hamsters (*34*). SV-40 is not related antigenically to polyoma virus. It does not induce tumors in mice and does not cause hemagglutination. It resists 1-4000 formalin, ether, and 60° C for 30 minutes. In the hamster, SV-40 infective virus disappears within 2 to 3 days after inoculation and reappears when the tumor develops, whereas, with polyoma virus, multiplication comes first and there is much less virus production by the time the tumor develops. SV-40 virus has recently been shown to cause some sort of transformation of human cells *in vitro*. More information is needed to interpret the significance of this finding (*35*). Another common virus, human adenovirus 12, has recently been shown by Trentin (*36*) to induce sarcoma in hamsters. The virus he used could be distinguished from polyoma and SV-40 by sensitivity of various tissue-culture cells, type of cytopathogenicity, virus neutralization tests with specific antisera, and electron microscopy.

MORPHOLOGY OF VIRUSES

Tumor viruses have the same diversity of sizes and shapes as regular viruses. The smallest known are the round papilloma wart-polyoma group measuring 30 to 50 mμ in diameter, and composed of a dense nucleoid surrounded by a single membrane (*37*). The largest are molluscum contagiosum and rabbit fibroma which are rectangular, belong to the pox group, measure 220 to 300 mμ in diameter, and contain a complex central nucleoid surrounded by multiple membranes. All the fowl tumors are associated with a single morphological type of virus particle, but in the mouse tumors 4 different types have already been described. Their significance is not clear; for example, type A particles have been seen in electron micrographs of 12 different neoplasms of the mouse.

CHEMICAL COMPOSITION AND SYNTHESIS

Only 2 tumor viruses have been prepared in a sufficiently pure state for chemical analysis. Shope papilloma (*38*) contains DNA and protein, and fowl leukosis virus contains ribonucleic acid, is associated with adenotriphosphatase activity, and is produced in close association with mitochondria. All the wart and papilloma viruses are associated with the cell nucleus, as shown by staining reactions. Polyoma and SV-40 viruses both contain DNA. Polyoma virus is formed in the nucleus and probably liberated by bursting of the nuclear membrane (*39*). Molluscum contagiosum, fibroma, myxoma, and pox viruses are produced in the cytoplasm, but it is not clear whether some portions may be produced in the nucleus with eventual assembly in the cytoplasm. The latter process is exhibited by the Lucké virus of the leopard frog and the herpes virus (*40, 41*).

POSSIBLE MECHANISMS OF VIRUS-INDUCED TUMOR FORMATION

Tumor formation induced by a virus could be due to a single new protein (*42*) synthesized in the cell under the genetic influence of the virus. In many cases, malignancy may be a secondary phenomenon after virus infection of the cell. For example, the rabbit papilloma is benign and euploid, whereas the carcinoma that develops from it is malignant and aneuploid (*43*).

At the time of change from papilloma to carcinoma, the virus may become integrated within the nucleic acid of the cell and lose the characteristics by which the virus is ordinarily identified. Another interpretation is that the virus may release the basal cells from normal regulatory control of proliferation, which thus favors the chance occurrence of chromosomal alterations, the carcinoma being initiated, once an aneuploid cell is produced that is capable of unchecked and aggressive multiplication. Such a cell is no longer dependent on the virus for escape from the normal growth regulatory mechanisms and may outgrow the virus in time. In other cases of virus-induced tumors, such as the Rous sarcoma and mouse and chicken leukemias, the growth appears to be malignant from the start. The finding of virus in the cells of mouse lymphatic leukemia induced by X ray suggests that all tumors produced by carcinogenic agents may be mediated through actuation of a latent virus (*44*).

On the basis of present evidence, there is no doubt that viruses are etiologically linked with some cancers of animals. However, viruses have not been demonstrated in a much larger group of cancers that arise spontaneously or after radiation and the application of certain chemicals. Generalizations are hazardous at this stage of knowledge. Each virus-induced tumor thus far studied has distinguishing properties and characteristics, and one can expect the detailed mechanics of induction to be different for each tumor-virus system.

TUMOR IMMUNITY

This is such a complex subject that it cannot be reviewed in the short time available. However, skin tumors offer some interesting points for speculation. The benign tumors of the skin associated with viruses are usually self-limited. After remission the animal is resistant to reinfection, and neutralizing antibodies can be demonstrated in the serum.

The viruses responsible for the induction of these tumors behave much like ordinary viruses, and resistance can be artificially induced by active or passive immunization. On the other hand, when the rabbit papilloma or other virus-induced tumors become malignant, the host tolerates their presence and antiviral immunity is of questionable value.

Thus, while proof of the viral etiology of a tumor is a great advance, such proof does not automatically explain the lack of immunity to malignant tumors. The basic problems remain: By what mechanisms does the tumor cell escape normal control of multiplication, and how are the normal defensive and immunologic mechanisms circumvented or blocked? Virus-induced tumors permit new quantitative experimental approaches to these problems. They also hold out hope for control of cancer without an understanding of these basic mechanisms, through application of three broad techniques, which have controlled many infectious diseases:

1) Identification of the virus and its elimination from the population through application of epidemiologic principles—as can be done with polyoma virus in mouse colonies.

2) Preparation of vaccines and enhancement of immunologic mechanisms.

3) Use of chemotherapy specifically directed to the virus or to the cellular changes induced by the virus.

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A VIRUS-INDUCED TUMOR IN PRIMATES[1,2,3]

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SUMMARY

A virus morphologically belonging to the pox group was studied. It produced lesions in the skin and internal organs of Asiatic monkeys. Histologically the lesions can best be classified as histiocytomas. Most African monkeys were resistant to the virus possibly because of previous contact and immunity, although no complement-fixing antibodies could be demonstrated in newly imported animals. Humans developed similar lesions upon virus inoculation. The virus appeared to be antigenically distinct from vaccinia and monkey pox virus.—NCI Monogr. No. 10: 447–458, 1963.

IN 1957, Bearcroft and Jamieson (*1*), at the laboratories of the West African Council for Medical Research in Yaba, near Lagos, Nigeria, noticed a tumor on the face of a rhesus monkey housed in an open pen. Similar tumors appeared in 20 rhesus monkeys occupying adjacent pens. One young dog-faced baboon was also affected, but other local West African monkeys in the colony remained intact. The tumors consisted of large pleomorphic cells, many of which contained inclusion bodies, which indicated a possible viral etiology. Samples of frozen tumor were sent to C. H. Andrews at the National Institute of Medical Research, Mill Hill, London. Andrews and co-workers (*2*, *3*) found that filtrates through gradocol membranes were able to induce tumors in rhesus monkeys. From filtration experiments and electron microscopic studies they concluded that a large virus possibly of the pox group is involved.

In this paper we will briefly report on the experience with this virus at the Roswell Park Memorial Institute. A number of detailed papers are in press or in preparation: Feltz *et al.* (*4*, *5*), Sproul *et al.* (*6*), Metzgar *et al.* (*7*), and Grace *et al.* (*8*, *9*).

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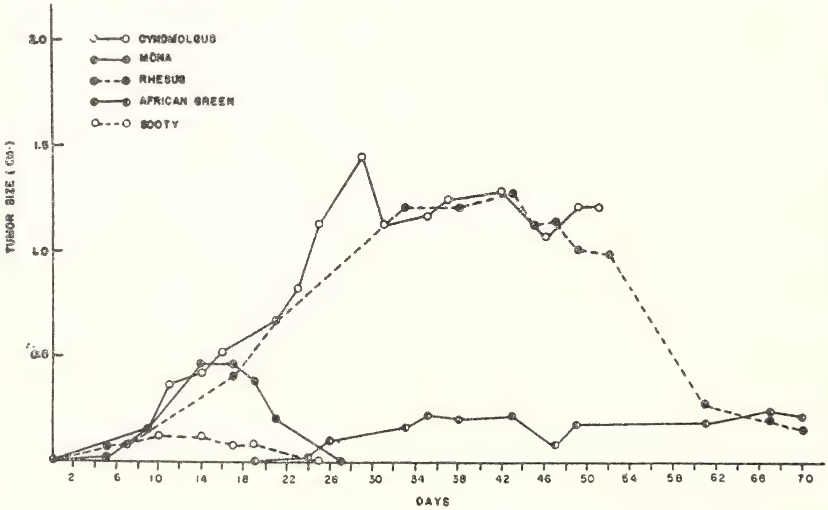
[4] We acknowledge the assistance in these studies of W. Kawinski, R. Shields, B.A., J. Bastian, and I. Mink, B.A.

SPECIES SPECIFICITY: EPIDEMIOLOGY

Homogenates from lesions excised from rhesus monkeys or cell-free filtrates thereof were inoculated intravenously, subcutaneously, intraperitoneally, intracerebrally, and intranasally into newborn and adult dogs, rabbits, guinea pigs, hamsters, rats (Wistar, Fischer 344), and mice (ICR/Ha, C57, C3Ht). No histologically characteristic lesions were produced. Occasionally small nodules which persisted for a few days and appeared to consist of inflammatory elements appeared at the inoculation site. Inoculation of viral filtrates onto chorio-allantoic membranes or into yolk sacs of embryonated eggs produced no visible lesions and reinoculation into monkeys did not induce tumors. Table 1 summarizes experiments on the species specificity in monkeys. In all of these experiments, homogenates of lesions excised from rhesus monkeys were inoculated subcutaneously into the shaven backs of monkeys in dilution series of 10^{-1} to 10^{-8}. In species where takes were obtained the experiments were repeated with cell-free filtrates. For purposes of this study "take" was defined as production of a lesion of at least 0.5 cm diameter which persisted for at least 3 weeks, showed a characteristic histologic picture, and when homogenized and inoculated into rhesus monkeys could induce tumor formation. Small inflammatory nodules were produced in many species. Text-figure 1 shows an experiment in which the homogenate of a lesion from a rhesus monkey was inoculated into 5 recipient animals. Only the rhesus and cynomolgus monkeys grew significant tumors. Regression starting around the 46th day occurred in the rhesus monkey. The cynomolgus monkey died apparently of superinfection. In the other species only small nodules developed.

It is apparent from table 1 that the virus seems to induce lesions in Asiatic monkeys but not in African and South American monkeys. The



TEXT-FIGURE 1.—Development of lesions in 5 species of monkeys after inoculation with tumor homogenates. Diameter of the largest section is given.

TABLE 1.—Susceptibility of various species of monkeys to Yaba virus

Origin	Species	Takes/No. inoculated
Asia	Rhesus monkeys (*Macaca mulatta*)	10/10, 10/10*
	Cynomolgus monkey (*Macaca irus*)	10/10, 8/10*
	Pigtail macaque (*Macacus nemistrinus*)	1/2
Africa	African green monkey (*Cercopithecus aethiops*)	2†/6
	Vervet monkey (*Cercopithecus aethiops pygerythrus*)	0/2
	Mona monkey (*Cercopithecus mona*)	0/2
	Sooty mangabey (*Cercocebus fuliginosus*)	0/2
South America	Squirrel monkey (*Saimiri sciurea*)	0/2
	Common white eared marmoset (*Callithrix jacchus*)	0/2
	Tamarin marmoset (Tamarinus‡)	0/2
	Pigmy marmoset (*Cebuella pygmaea*)	0/2

*Cell-free filtrate inoculated.

†Tumors appeared after a long latent period and reached maximal size 16 to 20 weeks after inoculation.

‡There are several species of Tamarinus between which we cannot definitely distinguish.

South American monkeys studied all belong to lower-order species and thus may not be strictly comparable to the African and Asiatic monkeys. The virus was originally discovered in a monkey colony in Africa where imported Asiatic monkeys developed tumors. The question arises whether the virus is endemic in certain areas of Africa and whether African monkeys develop immunity at an early age. The monkeys in the colony where the epidemic occurred were housed in open-air pens. During the last 3 years, about 250 monkeys in our colony were kept in the same room with Yaba virus-inoculated animals. With the exception of two instances where animals without record of inoculation developed typical lesions, no sign of contact infection occurred, even when infected and control animals were deliberately housed in the same cage. It is possible that anthropoid vectors were involved in the epidemic at Yaba.

While inoculating monkeys with virus suspensions, a technician accidentally punctured the web of his thumb with a virus-contaminated needle. Eventually a nodule developed which was excised. Histologically it was indistinguishable from the lesions in monkeys. There was an increase in complement-fixing antibodies. During an observation period of about 2 years since this episode no ill aftereffects were noted. Five volunteer cancer patients suffering from far-advanced incurable disease were inoculated with cell-free filtrates from lesions of monkeys (*8*, *9*). Typical lesions developed. Suspensions and cell-free filtrates of excised human lesions produced typical tumors in other human volunteers and monkeys.

EXPERIMENTS WITH VIRUS INOCULATION: IMMUNOLOGIC ASPECTS

Figure 1 shows the shaven back of a rhesus monkey 10 days after inoculation of a 10^{-1} to 10^{-8} dilution series of a viral suspension. Nodules were noted up to 10^{-3} dilution. Each dilution was injected in duplicate. On the 55th day the previously observed lesion attained maximal size (fig. 2) and there were signs of some necrosis. Smaller nodules appeared up to the 10^{-5} dilution and one of the larger tumors developed a "satellite" lesion beside it. On the 70th day (fig. 3) lesions became apparent up to 10^{-6} dilution, and most large tumors showed massive necrosis. On the 80th day (fig. 4) necrosis, hemorrhage, and superinfection were present in most lesions. The animal was in poor condition, ate little, and lost weight. Eventually it recovered and developed secondary tumors in different areas of the body. Figures 5 and 6 show such secondary tumors on the face and ankle of monkeys. Monkeys often went through 3 to 5 infectious cycles, new lesions developing in distant parts of the body about the time when the previous lesions regressed. Some animals died of superinfection and "cachexia." Others seemed to develop permanent immunity and did not exhibit further tumors.

Metzgar *et al.* (*7*) developed methods to test complement-fixing antibodies against Yaba virus. These antibodies disappeared rapidly from the circulation after regression or excision of the tumor. Metzgar *et al.* (*7*) found that animals with actively growing tumors and high antibody titers are resistant to additional virus inoculation. They concluded that the complement-fixing antibody may not react with intracellular virus but may inactivate and complex the newly inoculated virus. It is possible that there is a balance between virus released from the lesions and circulating antibody. Occasionally intracellular virus may persist until the antibody level becomes sufficiently low to permit distant infection, which thus allows for the recycling phenomenon described. Recently we have failed to produce lesions with virus inoculation in monkeys whose complement-fixing-antibody titer returned to baseline level following regression of tumors. We are searching for other immune mechanisms (neutralizing antibodies, tissue resistance factors, etc.) to explain this phenomenon. No complement-fixing antibodies were found in freshly imported African monkeys resistant to the virus.

Cross-resistance experiments were performed with Yaba virus, vaccinia virus, and the monkey pox virus of von Magnus *et al.* (*10*) and Prier and Sauer (*11*). Monkeys immune to the latter viruses developed typical lesions upon inoculation with Yaba virus.

Intravenous inoculation of cell-free filtrates of tumors into recipient monkeys caused the appearance of innumerable small spots all over the skin of the animal. During the following days the number of lesions decreased (fig. 7) but the remaining lesions continued to grow. Autopsy and histopathology of animals that expired revealed similar lesions in the lungs, and skeletal and heart muscle. Direct inoculation into the brain (2 animals) produced only nonspecific reactions. Inoculation into the liver produced only 1 lesion of 4 injections.

MORPHOLOGY

A detailed report on the pathogenesis and histogenesis of this disease is in preparation (*6*). Figure 8 shows a typical section on the 22d day after virus inoculation. Generally increased histiocyte numbers became apparent about the 3d day after virus inoculation. They enlarge, take polygonal shapes, and become multinucleated. Nuclei enlarge and nucleoli become very prominent. Mitotic counts increase and about the 5th day cytoplasmic inclusions become visible. Many histiocytes develop into elongated forms and become arranged into definite patterns, *e.g.*, spiral forms. By the 2d week many degenerating cells are visible, at the same time other cells continue to divide. Gradually, plasma cells and neutrophils invade the periphery of the lesion. Large number of capillaries grow into the tumor and cords of fibroblasts start to replace the degenerating histiocytoma cells. Regression occurs without central necrosis. Regressing tumors often ulcerate, hemorrhage, and become infected. However, many tumors regress without ulceration. Regional lymph nodes often become enlarged. Following intravenous injection there is usually generalized hyperplasia of the lymph nodes and spleen. Figures 9 and 10 show electron micrographs made by Dr. G. Owens. Masses of oval or rounded particles of high electron density are seen often in a paranuclear position. With higher magnification (fig. 10) these particles appear to have varying densities and internal structures. Many of these particles are brick-shaped. They are clearly similar to other pox viruses.

ATTEMPTS TO PRODUCE MALIGNANT TRANSFORMATION: EFFECT OF DRUGS

Members of paired dilution series inoculated into the dorsal skin of rhesus monkeys were treated with daily paintings of various concentrations of methylcholanthrene or dibenzanthracene in acetone or benzol. Control lesions were treated with solvents only. No malignant transformation was noticed. In many instances the carcinogen-treated lesions regressed early while the control lesions continued to grow. We are presently repeating these experiments. Similar results were attained in series of lesions into the center of which a small hole was burned every other day with electrocautery. Whole-body radiation before virus inoculation (200–400 rad) did not alter the course of the process; treatment with steroids was similarly ineffective.

Several established and new chemotherapeutic agents were tested for antiviral or antitumor effect (methotrexate, 6-mercaptopurine, 5-fluorouracil, 5-fluorodeoxyuridine, 5-mercaptouracil [AB 050 (*12*), NH_2, AB 103, and AB 132 (*13–15*), etc.]. The animals were treated daily by subcutaneous, intraperitoneal, or intravenous injections for 4 to 30 days with various doses. Tumor development, after subcutaneous inoculation of dilution series of a virus suspension, was compared with the growths resulting in control monkeys after inoculation of the same dilution series. The highest effective dilution, the speed of tumor development and regression, and the maximal size attained were compared. None of the drugs produced significant changes compared to the pattern of tumor development in the controls. These data will be reported in more detail elsewhere (*5*).

TISSUE-CULTURE STUDIES

For further studies it would be desirable to use cytopathogenicity in tissue cultures rather than tumor development in monkeys as a test. It would be helpful to be able to grow the virus in cultures. Table 2 summarizes our attempts to develop such methods. No frank cytopathogenicity was observed. Only in monkey heart cultures was occasionally a marginal increase of questionable significance in virus titer noticed upon reinoculation of dilution series into monkeys.

TABLE 2.—Yaba virus in tissue culture

Cell line	Cytopathic effect	Virus replication
Human conjunctiva (Chang)	—	—
Human embryonic skin (Gray)	—	—
D_6 human bone marrow (Berman, Stulberg)	—	—
D_{98} human bone marrow (Berman, Stulberg)	—	—
Human liver (Chang)	—	—
Human embryonic intestine (Henle)	—	—
HeLa human epidermoid carcinoma (Gey)	—	—
Monkey heart (Salk, Ward)	—	±
Monkey kidney (Primary)	—	—
Mouse embryo (Primary)	—	—

The Yaba virus appears to be an interesting model system which deserves further study. This brief preliminary report merely summarizes the present status of some of the studies at the Roswell Park Memorial Institute.

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PLATE 39

Yaba virus suspension inoculated subcutaneously in duplicate dilution series (10^{-1}–10^{-8}) into a rhesus monkey.

FIGURE 1.—Lesions following inoculation: 10 days.

FIGURE 2.—Lesions following inoculation: 55 days.

FIGURE 3.—Lesions following inoculation: 70 days.

FIGURE 4.—Lesions following inoculation: 80 days.

PLATE 40

FIGURE 5.—Secondary lesion on the face of rhesus monkey.

FIGURE 6.—Secondary lesion on the ankle of cynomolgus monkey.

PLATE 41

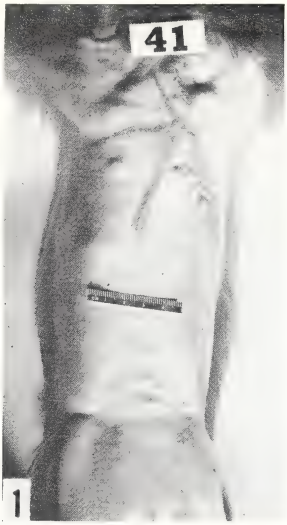
FIGURE 7.—Skin lesions following intravenous injection of cell-free filtrates of tumors.

FIGURE 8.—Histology of Yaba tumor 22 days after subcutaneous virus inoculation. The tumor extended to epidermis. Both rounded and spindle-form cells are visible. Many degenerating cells. At the same time active proliferation continues. There are 6 mitoses in this field.

PLATE 42

FIGURE 9.—Electron micrograph of intracellular Yaba virus.

FIGURE 10.—Electron micrograph of intracellular Yaba virus.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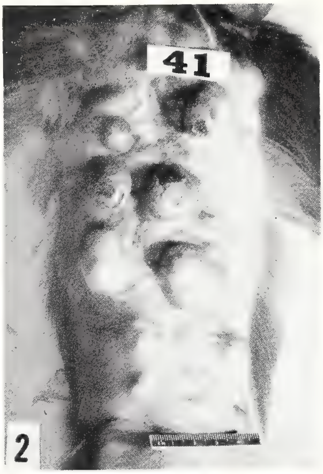
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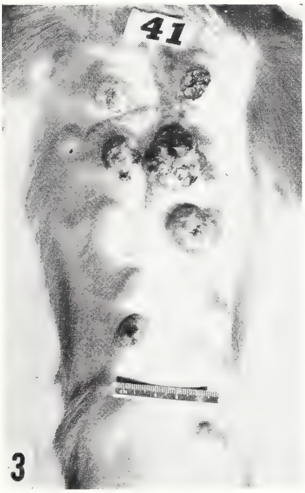
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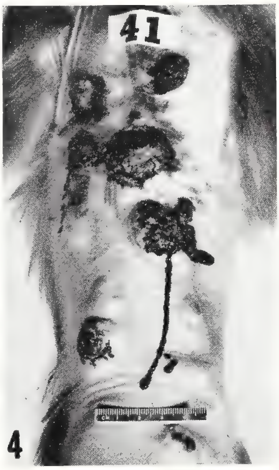
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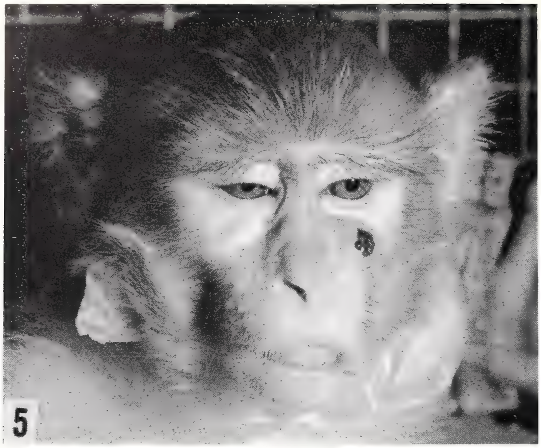

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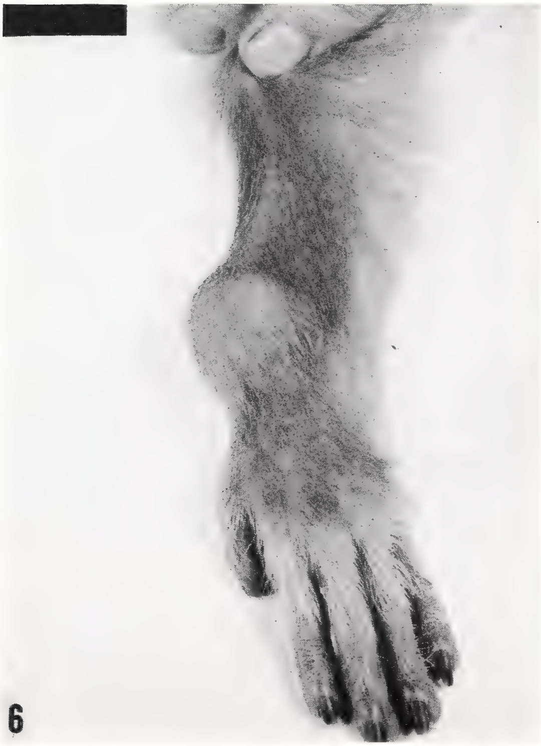

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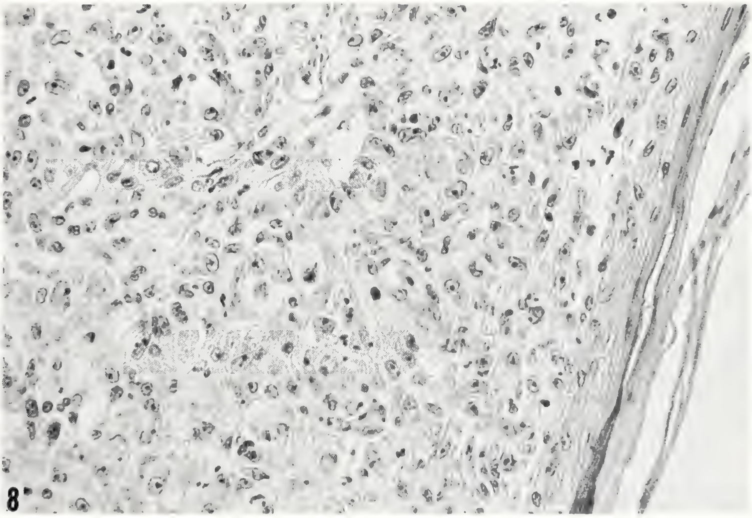
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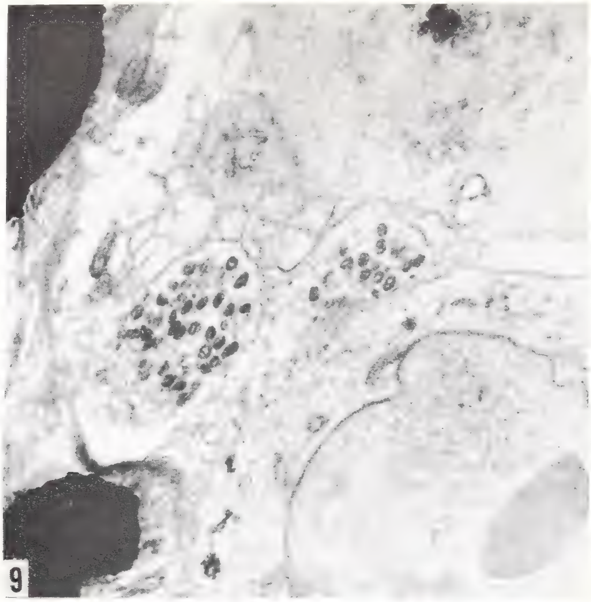
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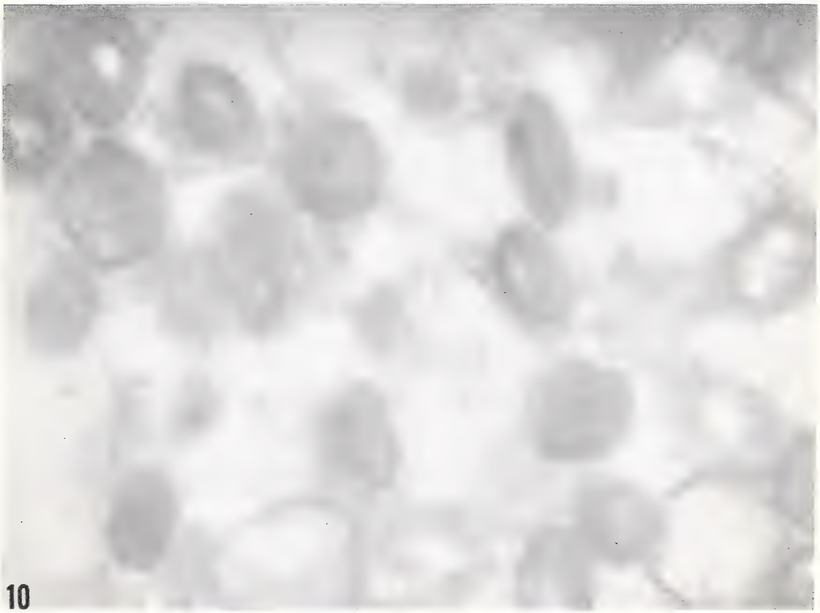
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AMBRUS *et al*

SKIN-APPENDAGE TUMORS INDUCED BY POLYOMA VIRUS IN MICE[1]

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SUMMARY

Unique benign tumors arising from hair follicles of mice inoculated with polyoma virus have been previously reported. In the present histopathologic study, tumors from 100 hosts of several inbred strains were examined. Although the tumors appeared to originate from follicle epithelium below the origin of the sebaceous glands, some showed ability of their proliferating cells to differentiate into sebaceous cells as well as into cells that keratinized and produced pigmented or nonpigmented structures resembling imperfect hairs. Glandular patterns resembling those seen in hidradenomas were often observed. The tumors are therefore more correctly viewed as organoid, developing from germinal cells that have variable capacity to differentiate toward any of the specialized cells of the pilosebaceous or sudoriferous apparatus, even though they occur in areas where sweat glands are not ordinarily present. These tumors are histologically distinct from polyoma-induced mammary tumors that frequently lie adjacent to them. They have not been seen to metastasize, and attempts to transplant them to polyoma-free, isologous, newborn, or adult hosts have not been successful. The influence of phase of the hair growth cycle on the genesis of these tumors needs clarification. Hair follicles adjacent to the tumors may be found in any phase of the cycle. That growth rate of the tumors may be affected by the phase of surrounding follicles is suggested by the observation that tumors in areas with hairs in resting phase were often smaller than tumors in the same animal, but located amid follicles in the growth phase. An exact counterpart of these murine skin-appendage tumors does not appear to have been observed in man. Possibly the closest homologue is the benign calcifying epithelioma of Malherbe.—NCI Monogr. No. 10: 459–488, 1963.

TWO TYPES of skin-appendage tumors occur commonly in mice as a response to polyoma virus (*1–3*). These are mammary tumors and tumors of the pilosebaceous apparatus. Each is a type *sui generis,* histologically distinct from other tumors arising at these same sites. One unusual instance of sweat-gland tumors occurring in a mouse that received polyoma virus has been reported (*1*).

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6-11, 1962.

[2] The author is indebted to Miss Marilyn G. Shaw and Mr. Willie D. Morgan for technical assistance with the animal and tissue-culture work, and to Mr. Gebhard Gsell for preparation of the photographs.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

As noted in a previous study (*2*), most of the tumors induced in mice by polyoma virus can be classified as tumors arising in appendageal structures, either of the skin or oronasopharyngeal and tracheal mucous membranes. Thus there are the tumors of all three major salivary glands, both sets of lacrimal glands, the accessory salivary and mucous glands of oropharnyx, larynx, and nasal mucous membranes; the tumors of dental epithelium, of the thyroid gland, and the thymic epithelial tumors. All of these have been previously described (*1–4*).

In this report, only the tumors of the pilosebaceous apparatus will be considered. Structural similarities between these tumors and benign calcifying epithelioma of man will be described. Some advantages provided by the polyoma-induced, skin-appendage tumors for investigation of the mechanism of action of the virus will be noted.

MATERIALS AND METHODS

The murine skin-appendage tumors were induced with a strain of polyoma virus originally isolated in this laboratory (*2*). The virus was passaged through P388 D_1 mouse lymphoma cells in tissue culture for 19 serial passages. Virus suspensions from various passages from the first to the nineteenth were used. Mice were inoculated subcutaneously within 12 to 72 hours after birth with 0.05 ml injected dorsally at the nape of neck. The dosage in terms of infectious particles was variable, and no attempt was made to correlate dose with response. Unpublished work has shown that the appearance of skin-appendage tumors is dependent not only on the viral dose, but also on viral strain variations (*5*).

Mice bearing tumors were mainly of strain C3H/Bi reared in a polyoma-free colony. Other strains and hybrids with these tumors and included in the study were: C3H/fg, DBA/2, AKR, RFM, $(\text{C3H/Lw} \times \text{AKR})F_1$, $(\text{AKR} \times \text{C3H/Bi})F_1$.

One hundred mice with pilosebaceous tumors at autopsy were used for the histologic study. They were selected in chronologic order from experiments testing the oncogenic potency of virus pools.

Tissues were routinely fixed in Zenker-formol and stained with hematoxylin and eosin. Skin blocks containing multiple tumors from 2 mice were fixed in 10 percent formalin for fat stains with Oil Red O. Multiple tumors from 6 mice were stained with Giemsa, the Ziehl-Neelsen acid-fast method, the Feulgen method, pyronin-methyl green, Lillie's ferrous iron method for melanin (*6*), von Kossa's method for calcium (phosphate), and Glenner and Lillie's method for tyrosine (*7*).[4] Serial sections were studied from several tumors of a single animal.

Sections and paraffin-embedded blocks from 21 specimens of benign calcifying epithelioma were kindly made available by Dr. Benjamin Highman of the Laboratory of Experimental Pathology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland. Some of these tumors were reported by him in a previous study (*8*). Besides hematoxylin and eosin stained sections on all of these, sections of 6 specimens were studied after being stained with the same special methods listed for the murine tumors.

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[4] Dr. G. Glenner, Laboratory of Experimental Pathology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md., kindly performed the diazo-coupling procedure for tyrosine on these sections.

OBSERVATIONS

General

Brief descriptions of the structure and histogenesis of these tumors and certain lesions that represent either abortive tumor development or non-neoplastic response to the virus (*2, 3*) have been published previously. Experience with increased numbers of the tumors and more extensive histologic study permit more complete description and modification of some earlier impressions. More accurate reconstruction of the processes involved in tumor development and growth is also possible. Although the present study is based on necropsy study of 100 mice bearing skin-appendage tumors, the tumors were almost invariably multiple and tended to occur in clusters, so that each section usually provided a cut through several, and often dozens, of individual tumors. Multiple skin blocks were taken from most of the mice, with the result that the total number of tumors and related lesions studied was in the thousands.

Figures 1 and 2 show the external and internal appearances of the integument of a C3H/Bi mouse bearing many skin-appendage tumors, as well as multiple tumors of the salivary glands. In this example, many of the skin-appendage tumors were large enough to be seen externally without the hair being shaved. The external coat was irregularly lumpy or cobblestoned. In many mice, however, skin-appendage tumors were not detected until the inner aspect of the skin was exposed. Then, even minute, pinhead lesions like those in figure 2 could be seen because the pigment within them produced a sharp contrast to the adjacent skin. In unpigmented strains of mice the smaller lesions were difficult to detect. They could be found more readily under a strong light beam directed obliquely across the exposed inner surface of the skin. Reflections from the raised lesions then caught the eye.

Skin-appendage tumors have been observed in all the hairy parts of the skin, with the possible exception of the pinnae of the ears. I have seen 1 animal with histologically confirmed multiple skin-appendage tumors on the tail. Stanton has reported them in the follicles of the vibrissae (*3*). The majority of the tumors are situated around the trunk, but they also occur less frequently on the legs and face. Their heaviest distribution seems to follow roughly the pattern of mammary-gland distribution, though obviously this correlation is not an absolute one. Neither is there a simple correlation of tumor frequency or size with hair length, as the tumors are not most numerous or largest on the back, though they often are found there. It is curious that large skin-appendage tumors are often found in juxtaposition to polyoma-virus-induced mammary tumors (table 1). The basis for this relationship may rest simply on the normal anatomic proximity of mammary glands to the skin.

TABLE 1.—Features of polyoma-virus-induced skin-appendage tumors

Feature	Percent of mice showing (total 100 mice)
Pseudo hair follicles	93
Pigment cells	93
Nuclear "vacuolation"	85
Intranuclear inclusion bodies	77
Interstitial basophilic lakes	26
Sebaceous cell proliferation	25
Gland formation	21
Associated with mammary tumor	21
Completely regressed lesions	3
Calcification	2
Ossification	0
Invasion or metastasis	0

Histopathology

The response of the pilosebaceous apparatus to infection by polyoma virus followed a variety of patterns. Many of these could be found within a single section cutting across multiple lesions. It was possible to classify individual tumors or lesions according to size, the relative amount of proliferative activity, and the direction(s) of differentiation displayed by cells stemming from the proliferating process. Thus small, intermediate, and large lesions were found in which proliferative activity at the time of examination was minimal or absent. On the other hand, small, intermediate, and large lesions were found in which abundant proliferative activity was clearly evident. A given lesion could thus be classified as "active" or "inactive," much as one can classify hair follicles as growing or resting. Furthermore, in all the larger lesions and in most of the smaller ones, evidence could be found of past or present cellular differentation in one or more of three directions: (*a*) toward keratinizing cells organized in patterns resembling hair follicles in growth phase; (*b*) toward sebum-producing cells, often arranged in patterns simulating sebaceous glands; (*c*) toward columnar cells forming fluid-containing, glandlike spaces.

Other features of the tumors or lesions deserve mention, though they are of secondary significance. In all except the very smallest lesions, and particularly in the larger ones, whether active or inactive, necrotic central masses formed the majority of the substance of the lesions. The central necrosis was of two distinct types, often intermixed in a single lesion. One type of necrotic material was strongly eosinophilic and had the features of central tumor necrosis that results from impaired vascular supply. The other type of necrosis was characterized by a refractoriness to staining by either eosin or hematoxylin, by an acid-fast affinity for basic fuchsin, by birefringence in polarized light, and by a weak reaction for tyrosine, thus having the features of keratinizing hair shafts. Further morphologic details of this type of necrosis will be given later.

A third type of necrosis was apparently caused directly by viral parasitization of follicle epithelium and was observed as death and disintegration of individual cells in areas where neighboring cells contained intranuclear inclusion bodies.

Exceptional cases (2 of 100) showed calcium deposits in areas of necrosis. Where these were present, the necrosis appeared long standing. The calcification occurred within necrotic foci of the massive, eosinophilic type.

Another feature of some lesions was the presence of a granulomatous inflammatory reaction around their periphery. This reaction was composed chiefly of macrophages, foreign body giant cells, lymphocytes, and plasma cells. Such reactions were more often found around completely inactive and necrotic lesions than around actively growing ones. In many instances where the inflammatory reaction was especially pronounced, the saccular, necrotic structure had ruptured, with keratinaceous material released into the tissues.

To provide a concept of the morphogenesis of the various types of lesions representing the responses of the pilosebaceous apparatus to polyoma virus, the following reconstruction is presented.

The first evidence of response was seen as an enlargement of the follicle or some portion of it, associated with hypertrophy of some of its cells (figs. 3, 4, and 5). In most lesions at this stage, the cellular hypertrophy was accompanied by intranuclear inclusion bodies in some of the enlarged cells. Necrosis of individual cells was also seen, this apparently being the ultimate fate of cells containing inclusions. The inclusion bodies were single or multiple, generally sharply outlined, and tended to be spheroidal (fig. 6). In hematoxylin-eosin stained sections, they were characteristically surrounded by distinct, clear zones. Another type of nuclear lesion, resembling nuclear vacuolation or "ballooning," was also sometimes present. This lesion has often been noted in other polyoma-virus-infected tissues (*1–3*). The lesion consisted of an approximately spherical and distinctly demarcated area within the nucleus, in which there was very little stained material. Under high magnification, small particles, generally eosinophilic, could barely be discerned within the lesions.

Feulgen and pyronin-methyl green stains were done on sections from several tissue blocks. The intranuclear inclusion bodies gave a positive Feulgen reaction. With pyronin-methyl green, they generally stained dark blue, though some inclusions in cells apparently about to disintegrate showed a distinct reddish tint.

The impression was gained that not all portions of the pilosebaceous apparatus responded with equal readiness to the virus in the fashion described. Cells of the bulb, the hair matrix, and the inner hair sheath were most commonly involved (figs. 3, 4, and 5). It could not be positively established that cells of the external sheath ever developed inclusion bodies. Cells of the sebaceous glands did not undergo the type of hypertrophy, with nuclear inclusions, seen in the matrix and inner hair sheath of the follicles. These points are of interest because some of the later, larger tumors showed areas with morphologic characteristics of external sheath as well as definite sebaceous cells.

From the stage of cellular hypertrophy associated with nuclear lesions and cell death, the responding follicles proceeded in one of two main directions. In some examples, the cytolytic process predominated to the extent that the follicles were eventually completely killed and remained only as solid or cystic clumps of dead epithelium and keratin, surrounded by foreign body reactions (figs. 7 and 8). Residues of this small size perhaps represent primary necrotizing responses rather than regressed tumors, as it is uncertain that a definite proliferative stage was ever established in them.

The other type of response was one in which proliferation predominated (figs. 3, 4, 9, and 10). The proliferative process sometimes seemed to originate from the bulb of a follicle (fig. 4). In other instances (fig. 9) it appeared to arise from the external sheath cells high up on the follicle, adjacent to the sebaceous glands. When growth took place close to the junction of follicle and sebaceous gland, the sebaceous cells were enveloped by the proliferating mass. Bulbous outgrowth from the side of a follicle sometimes resulted in retraction of the upper part of the follicle and the sebaceous gland down toward the outgrowth, severing the connection of the follicle with the epidermis (fig. 9).

When an active proliferative phase of the response had become established, inclusion bodies and the vacuole-like type of nuclear lesion were no longer found. Mitotic figures became very numerous, and, in general, the most actively proliferating epithelium resembled that normally seen in the bulb and matrix of hair follicles in the growth phase.

Even when still small, the active proliferations developed central necrotic areas (figs. 3, 5, and 9). As the nodules enlarged, the central necrotic masses increased proportionately, while the peripheral rims of actively growing epithelium tended to retain a thickness limited to about 30 cell layers or less (figs. 10, 13, and 16).

Apparently, active growth could cease even in large, well-established tumors. Figure 11 shows a cluster of "tumors," most of which have become completely necrotic. Some contain calcium deposits (fig. 12) and granulomatous reactions surround many. Only a few still retain viable and proliferating epithelium.

The observation of apparently spontaneous tumor regression prompts the question as to whether growth of the tumors is influenced by the hair growth cycles, or vice versa. This question was kept in mind while the sections were reviewed, but a clear answer could not be obtained. As shown in figures 13 and 14, it could be demonstrated that normal hair follicles overlying active tumors could be in either the growth or the resting phase. This evidence argues against the possibility that the presence of an appendageal tumor might continuously stimulate or inhibit the growth of adjacent follicles.

On the other hand, it was several times noted, in long sections through the skin of a given animal, that tumors were larger in areas where the normal follicles were in growth phase than in contiguous areas where the normal follicles were in resting phase (figs. 14 and 15). This is merely an interesting observation, not believed to be sufficient evidence to conclude that growth of the tumors is controlled by the same factors (still unknown) that control the growth cycles of normal follicles.

Differentiation Within Tumors

One of the most impressive features these tumors showed was a disposition of the epithelium to differentiate along one or more of 3 major pathways, which have been defined as toward 1) keratin production, 2) sebum production, and 3) gland (sweat?) formation.

All three types of differentiation were sometimes seen within a single tumor, as illustrated in figure 22. However, as indicated in table 1, the most common form of differentiation was keratinization, with formation of structures resembling imperfect hairs. This occurred in almost all animals bearing these tumors. The only exceptions were animals whose tumors were all very small and hence were quantitatively limited in their opportunity to display differentiative capacities.

Figures 16 to 19 illustrate the process of "pseudo-hair formation" within the saccular tumors. Figures 16 and 17 show pseudo-hair formation in tumors from a nonpigmented (AKR) and a pigmented (C3H/Bi) mouse respectively. The phenomenon was the same in these strains, except that pigment-containing dendritic cells were lacking in lesions in AKR mice as in other strains with white coat.

Pseudo-hair formation appeared to occur as follows: First, an invagination of the stratum of germinal epithelium lining the tumor sac appeared. Capillary blood vessels were either carried in with or took an active part in initiating the invagination. The result was that a short, cylindrical structure with components analogous to the bulb, matrix, and dermal papilla of a hair follicle was formed, with the distal end of the incomplete follicle directed toward the center of the saccular tumor. In a sense, a structure of this type was much like a hair follicle turned inside out, or by some other means stripped of its internal and external sheaths. Without the sheaths and dermal tissues, there was no mechanism for compressing the keratinizing cells derived from the matrix into a compact hair shaft. Actually, in one exceptional example shown in figure 19, a pseudofollicle showed evidence of differentiation of some of its epithelium into internal sheath structures. This "follicle" could not pass as completely normal, however, because capillary vessels were present in the center of what would otherwise appear to be the lower part of a hair shaft.

Figures 17, 18, 19, and 20 show that, in pigmented mice, dendritic pigment cells became prominently aggregated around the central vascular loops of the pseudofollicles. This added further to the similarity between the pseudo and normal follicles. H_2O_2 bleaching and the ferrous iron method of Lillie for melanin demonstrated that this pigment was actually melanin.

As the papillary invagination of tumor epithelium and its central vessels extended further into the necrotic material of the saccular lesion, keratinization of the epithelium took place. This keratinized epithelium never became as hyalinized and amorphous as the keratin of normal hair shafts, presumably because of the lack of a compressing mechanism. However, it displayed birefringence in polarized light (fig. 20), was acid-fast when stained with fuchsin (fig. 21), and gave only a weak or negative tyrosine reaction (fig. 29). These properties are all shared with normal hair-shaft keratin in the mouse.

The origin of the melanin-containing dendritic cells in the pseudofollicles is problematic. It is presumed that they were derived from the pigment cells previously associated with the normal follicles. They were so numerous as to suggest that they must have undergone some proliferation, but their morphology and behavior did not indicate they were neoplastic. Their attraction to the central, perivascular regions of the pseudofollicles was striking (figs. 17, 18, 19, and 20), and reflected a remarkable migratory capacity. The lack of pigment within tumor epithelium suggested that the melanoblasts were unable to transfer pigment to the tumor cells.

Table 1 records that many tumors contained foci in which there was sebum production. Sebaceous cells in these foci appeared to differentiate from cells of the nonspecific germinal type that formed the peripheral, viable stratum of the saclike tumors (fig. 23). There was no special situation that appeared to favor their appearance, but frequently cells nearby could be found in a double-rowed palisaded arrangement simulating external hair sheath.

Although the sebaceous cells were morphologically similar to normal sebaceous cells, their large numbers and the obviously high rate of mitotic activity of the cells from which they were stemming spoke against the possibility of their being trapped elements of normal sebaceous glands. As there was no mechanism for release of sebum to the exterior, pools of oil became enclosed within the tumors. In paraffin-embedded sections these appeared as large, empty spaces.

Glandlike structures were found in one or more tumors in 21 percent of the animals studied (table 1). Whether these glandlike structures represented tumor mimicry of sweat glands is uncertain. The fluid contained in the larger spaces was colloid, staining moderately intensely with eosin. In some, many intact red cells were present. The fluid-containing spaces were not blood-vascular, however, as it was quite easy to trace their development through stages of enlargement from very small interstitial spaces that could be first recognized as little clefts or lakes scattered about in solid masses of unspecialized tumor cells. The material contained in these smaller spaces was generally basophilic and formed thready, weblike configurations. As the lumens became larger, their contents took on the eosinophilic colloid character, and, at the same time, the cells lining the spaces became arranged in double rows (fig. 24), much like the epithelium of sweat glands.

Transplantation

Our attempts to transplant these tumors to histocompatible secondary hosts have been unsuccessful. Subcutaneous transfers of tumor mince were made with tumors from C3H/Bi donors. The recipients were 8 adult male C3H/Bi mice and 9 (AKR × C3H/Bi)F_1 newborn mice, all from a polyoma-free colony. None of the 17 recipients developed tumor at the site of transfer after observation periods of 3 to 7 months. One animal that received the tumor in the newborn period developed multiple tumors at various sites susceptible to tumor induction by polyoma virus: salivary glands, thymus, renal medulla, and skin appendages. This was interpreted to mean only that a large dose of virus had been transferred to this animal in the tumor transplant. Possibly the transplant had included lesions containing the intranuclear inclusion bodies, which are thought to contain large amounts of virus.

Comparison With Calcifying Epithelioma in Man

Benign calcifying epitheliomas of Malherbe (*9*), or pilomatrixomas (*10*), have been well described in the literature (*8–12*) and their features will not be reviewed in detail here. The purpose of considering them is to direct attention to certain resemblances between them and the skin-appendage tumors induced by polyoma virus in mice (table 2). There are also differences between these two types of tumors, so that on a morphologic basis it is hardly possible to consider them precisely identical. However, many, though not all, of those who have studied calcifying epitheliomas have inclined toward the opinion that they originate from hair follicles or sebaceous glands or both. The persistence of some uncertainty concerning the histogenesis of calcifying epitheliomas is a fair indicator that it is more difficult to trace their origin than that of the polyoma-induced skin-appendage tumors of mice.

As illustrated in figures 25, 26, and 27, the general features of calcifying epithelioma and the polyoma-induced skin-appendage tumors are much alike, at least in the larger and older examples of each. Small, early lesions of the type seen in mice have probably rarely, if ever, been encountered in the human material. In both types, the tumors are saclike structures with variable amounts of viable epithelium lining all or parts of the periphery and extending in folds or papillae into the central parts of the sac. In both tumors, the epithelium undergoes keratinization, generally without formation of basophilic keratohyalin granules, and thus simulates keratinization in hair shafts. In the human tumors, trichohyalin granules occur commonly. Melanin pigment may be present in both, though it is certainly more common and prominent in the tumors of mice. Calcium deposits occur in both types of tumor, but are much more

Table 2.—Comparison of polyoma-induced skin-appendage tumors with benign calcifying epithelioma

Features	Polyoma appendageal tumors	Benign calcifying epithelioma
Distribution	Trunk, head, limbs	Head, neck, limbs
Age (not comparable because of differences in lifespan)	1–4 Months (inoculated at birth)	All ages, often in childhood
Location	Dermis and subcutaneous tissue	Dermis and subcutaneous tissue
Multiplicity	Nearly always multiple	Often multiple
Invasion or metastases	Not observed	Claimed rarely
Calcification	Rarely	Commonly
Ossification	Not observed	Occasionally
Keratinization	Regularly	Regularly
Melanin deposits	Regularly	Occasionally
Sebaceous gland formation	Commonly	Rarely
Gland formation	Commonly	Not reported
Pseudo-hair follicle formation	Regularly	Described (*10*)
Foreign body reaction	Occasionally, only mild or moderate	Commonly, and very marked
Self-limited growth	Occasionally	Commonly
Association with multiple tumors in other sites	Regularly	Not reported
Intranuclear inclusion bodies	Commonly	Not reported

common in calcifying epithelioma. This difference may be related to the much longer duration of the human lesions before removal and examination. Totally necrotic lesions of both types may occur, giving the appearance of "self-limited growth." Granulomatous reaction occurs around both types of lesion, but is more the rule and is much more prominent in benign calcifying epithelioma. This, as well as the ossification that has frequently been noted in calcifying epitheliomas, may also be related in part to the longer duration of the human tumors.

The major differences between the polyoma-induced appendageal tumors in mice and calcifying epithelioma in man are the absence of viral intranuclear inclusion bodies in the latter and the much less obvious evidence of differentiation toward hair follicles, sebaceous glands, or sweat glands in the human tumors. Thus these have a less organoid structure than the murine tumors under discussion. Finally it may be added that the keratinized epithelium in both types of tumor is birefringent in polarized light, but the keratin in the human lesions is more eosinophilic and gives a stronger tyrosine reaction than that in the murine tumors (figs. 28 and 29). This might be interpreted as further evidence of a lesser capacity to produce hairlike structures by the human tumor type, or it might be interpreted as a difference related to species differences in keratin formation.

DISCUSSION

These unique tumors of the pilosebaceous apparatus of mice are of special value to investigators for several reasons. First they are of interest from the point of view of the embryologist, anatomist, and dermatologist because they represent a response by a structure whose normal and abnormal development and functions are coming to be better understood as a result of the studies of recent years (*13–15*). Though it may not be possible to draw conclusions about normal hair development, growth, and regeneration from a knowledge of these tumors, nevertheless one gains from such knowledge a broader insight concerning the capabilities of this skin appendage. Such insight is likely to be followed by new experimental approaches to questions concerned with factors influencing normal growth and differentiation and the manner in which such factors may also influence tumor development.

The tumors are interesting to virologists because they demonstrate in a spectacular way an array of changes that a virus can initiate in the lives of cells and complex cell societies. Necrosis, proliferation, and variable effects on special function are the results of infection by a single virus species.

How can one agent produce these variable effects? It seems probable that the varied responses will be explained on the basis of the interactions between virus and cells that are in variable states functionally, and, therefore, presumably structurally. It seems reasonable to suppose that an epithelial cell in a hair follicle in the resting phase might respond differently to the polyoma virus than would the same cell in a follicle in growth phase. The relationship of an infected cell to its normal source of nutrition and control might radically affect the interactions between cell and virus. As evidence of this in another virus-tumor system, we have the important observations of Noyes and Mellors (*16*) on the rabbit papilloma, showing that the basal proliferating cells apparently support little or no viral replication, while the keratinizing cells rapidly become filled with complete virus particles. This situation is reasonably analogous to that seen in the early stages of change in hair follicles infected with polyoma virus. Here the maturing cells of the hair matrix often contained intranuclear inclusion bodies, while the cells of the external sheath did not. More specific information is needed regarding the viral content of cells of different types and in different morphologic phases of response.

Mechanisms of viral tropism become especially interesting when the pattern of tumor development in response to polyoma virus is considered. This pattern seems to vary according to the strain of virus and also according to the strain of mouse, but, with the respective strains of each used in this study, the organs most commonly involved are parotid, submaxillary, and sublingual salivary glands, lacrimal glands, thymus, submucosal glands of the nasal mucous membranes, oropharynx, and larynx, dental organs, mammary glands, hair follicles, thyroid gland, adrenal medulla, and bones. It was previously noted that hair follicles, mammary glands, and tooth organs are appendages of ectodermal origin, as are probably the lacrimal and salivary glands. Thyroid gland and thymus may also be thought of as appendageal structures, though of endodermal origin. If one can minimize the importance of drawing a sharp line between ectoderm and endoderm, it can be said that nearly all (adrenal medulla, renal medulla, and bone excepted) the sites of tumor development in response to polyoma virus in mice can be categorized as appendages of the skin or of the respiratory-enteric canal above the esophagus.

Where is the common denominator to explain the predisposition toward tumor response in these sites? There is still no answer to this question. However, I would like to make note of a remarkable condition in man, which may be either a clue or a very attractive "red herring" in this mystery.

This condition is known as ectodermal dysplasia [*see* (*17, 18*) for review]. In its severest form (anhidrotic ectodermal dysplasia) it is characterized by a complete or partial failure of development of the hair, sebaceous glands, sweat glands, teeth, lacrimal glands, salivary glands, and submucosal glands of the nasal passages and oropharynx. Sometimes (more often in males than females) the nipples and mammary glands are missing. The skeleton is generally frail and slight, and abnormal bone development is sometimes further reflected by short or absent phalanges, saddle nose, and poorly developed jaws and sinuses.

This hereditary syndrome is reported to be transmitted through a single gene abnormality, sometimes as a sex-linked recessive but at others as a straight dominant. It is brought into this discussion because of the remarkable (though not complete) overlap of organs involved in this syndrome and organs involved by tumors in mice inoculated with polyoma virus. Table 3 compares the organ involvement, in what might be called the polyoma-tumor syndrome of mice, with organ involvement in the syndrome of anhidrotic ectodermal dysplasia of man.

What is the significance of this observation? The most tempting interpretation would be that, in the mouse, development of the tissues

TABLE 3.—Comparison of sites involved in polyoma-tumor syndrome and in anhidrotic ectodermal dysplasia

Organ or tissue	Tumors in C3H/Bi mice inoculated with polyoma virus	Defective development in anhidrotic ectodermal dysplasia in man
Salivary glands	+	+
Lacrimal glands	+	+
Nasal submucosal glands	+	+
Oropharyngeal and tracheal submucosal glands	+	+
Dental primordia	+	+
Pilosebaceous apparatus	+	+
Mammary glands	+	+
Sweat glands	+ (Reported by S. E. Stewart)	+
Bone	+	+
Harderian glands	+ (Rare)	
Thymus	+	−
Thyroid	+	−
Adrenal medulla	+	−
Renal medulla	+	−
Subcutaneous connective tissue (nerve sheath cells?)	+	−

affected by polyoma virus is controlled by a gene comparable to that apparently responsible for development of nearly the same group of tissues in man. If true, this could have much bearing on concepts of tumor genesis with polyoma virus. It would suggest that polyoma virus (a DNA virus), in the process of converting a cell to a "neoplastic" one, locates itself on an "ectodermal appendage gene." It would also mean that, for the virus so to locate itself and induce tumor development, the ectodermal appendage gene would have to be actively engaged in, or "determined" toward, directing cell function, in concert with particular epigenetic factors. Otherwise, there would be no reason why *any* mouse cell should not respond to the virus by proliferating into a tumor. This, from experience, does not happen, at least *in vivo.* Only the cells of certain tissues or organs are able to realize a capacity to grow into tumors in response to the virus. Thus, the tumor-inducing action of polyoma virus would appear capable of expression only in the presence of very special genetic-epigenetic interaction.

It is unfortunate that in mice we do not have a single gene abnormality producing a syndrome like anhidrotic ectodermal dysplasia in man. Mice with such a genotype would furnish ideal material to study the interaction of polyoma virus and host-cell genome. Lacking this ideal tool, we do have genetic variants in the form of "hairless," "rhino," and "naked" mice (*19*). Though the phenotype in each of these variants does not truly lack hair-forming structures, nevertheless hair formation is abnormal, and it seems probable that the response to polyoma virus by mice bearing these genes would differ from that of mice with normal hair. Here lies an area begging for exploration. Another area equally important and equally untouched as yet is that concerned with the influence of development and phase of the growth cycle on the infectivity and responsiveness of hair follicles to polyoma virus.

Finally, those who occasionally observe benign calcifying epitheliomas in the clinic might consider the advisability of trying to identify some of these rare tumors before fixation and of attempting to isolate a viral agent. There can be no assurance that one will be found, for the existence of structural similarity (not complete) between the polyoma-virus-induced skin-appendage tumors of mice and calcifying epitheliomas of man can hardly be accepted as a certain indicator of viral etiology of the human lesion. There is some precedent, however, on which to build enthusiasm for such an approach, if one wishes to consider human warts and the Shope papilloma of rabbits.

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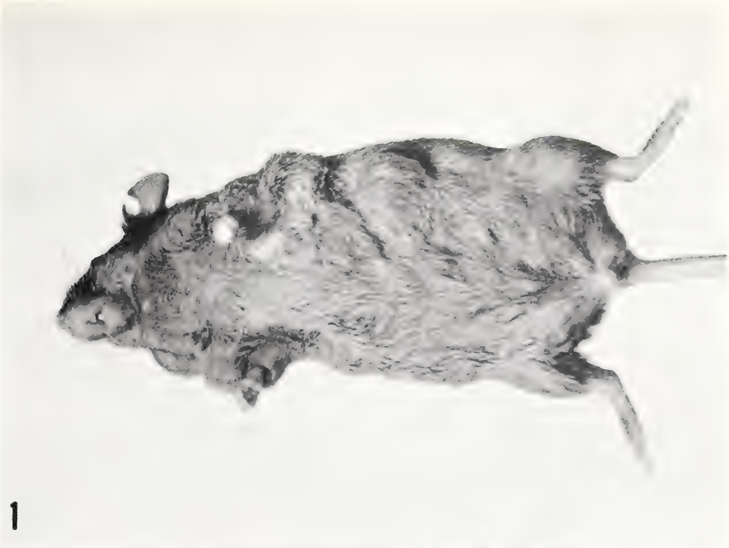

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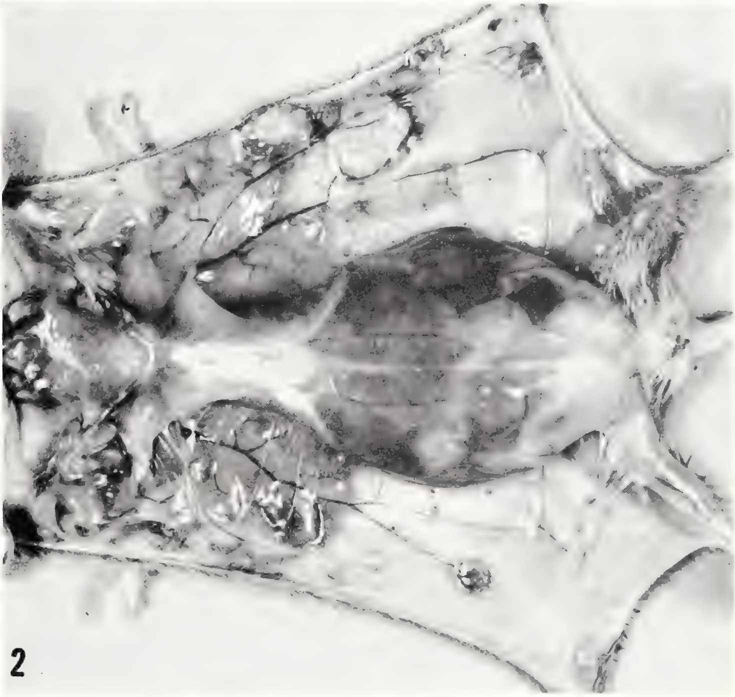

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FIGURE 1.—C3H/Bi female mouse, 3 months old, inoculated on day of birth with polyoma virus. Large skin-appendage tumors are visible along left flank.

FIGURE 2.—Same animal shown in figure 1, with skin reflected. Large tumor over left flank contains little pigment, while that below right axilla is dark with melanin. *Note* also several intermediate-sized tumors and numerous small ones appearing as tiny black spots.

PLATE 44

FIGURE 3.—Section of small tumor developing in lower two thirds of hair follicle. Cross section of hair shaft is in *uppermost* part of lesion. Small focus of necrosis at *deepest* portion. Between these points hair canal is filled with proliferating cells closely resembling hair matrix cells. To *left* is a cross section of enlarged follicle that has undergone nearly complete necrosis. Hematoxylin and eosin. × 150

FIGURE 4.—At *left center* small, actively growing tumor is developing from base of hair follicle. At *lower center*, another follicle shows enlargement and irregular shape, but nuclei of most cells show "ballooning" viral lesions or inclusion bodies. Hematoxylin and eosin. × 125

FIGURE 5.—Small lesion containing mixture of proliferating and dying cells occupies lower portion of a hair follicle. Cells at base of follicle contain intranuclear inclusion bodies and are undergoing necrosis. Higher up, external sheath encloses nests of proliferating epithelium filling hair canal. Hematoxylin and eosin. × 150

PLATE 45

FIGURE 6.—Intranuclear inclusion bodies in polyoma-virus-induced lesion of hair follicle. "Ballooned" nuclei also present, one at *lower center*, another at *upper left*. Hematoxylin and eosin. × 610

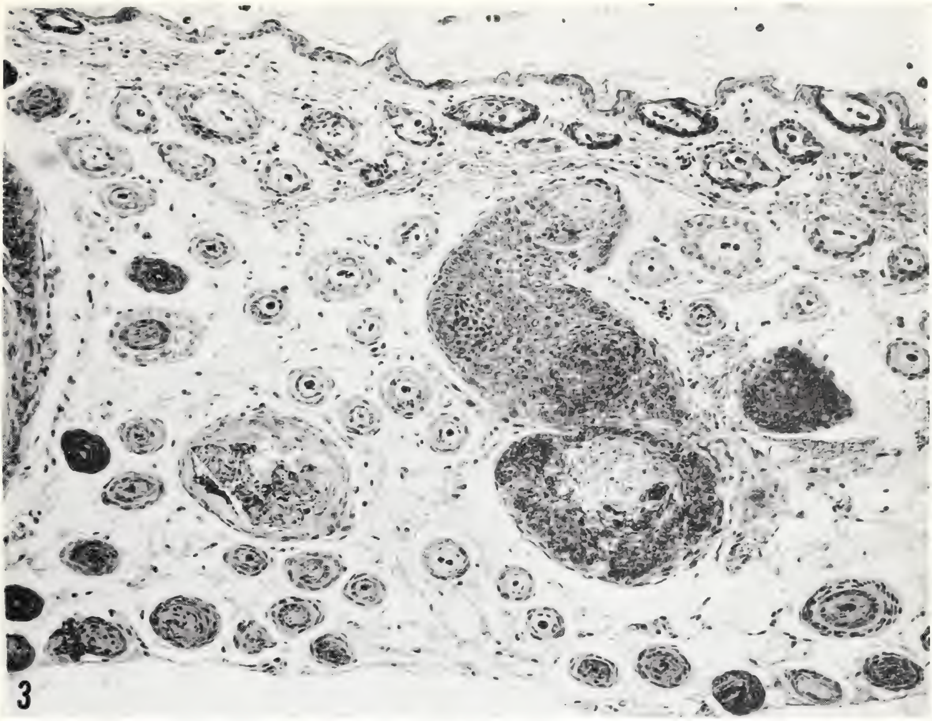
FIGURE 7.—Enlarged hair follicle in which reaction to virus has been predominantly necrotic. Viable hair matrix cells persist at base of follicle. Soft keratinaceous material fills hair canal. Follicle sheaths no longer identifiable. Hematoxylin and eosin. × 108

FIGURE 8.—Completely necrotic hair follicle lesions adjacent to several unaffected follicles. *Note* nonstaining necrotic epithelium (shadow or "mummified" cells) partially surrounded by calcific deposits (dark, amorphous areas). Foreign body giant cells are in contact with dead epithelium of lesion at *upper left*. Hematoxylin and eosin. × 135

PLATE 46

FIGURE 9.—Section of tumor springing from upper part of a hair follicle. Epidermal surface is out of the field, to *right*. *Note* continuity of tumor with external sheath of follicle. Tumor cells are beginning to envelop sebaceous glands (clear cells at *right center*). Pigment cells form black collar around branch of Y-shaped vessel entering tumor at region of attachment to follicle. *Arrow* indicates part of original hair shaft, which has been retracted and surrounded by tumor. × 108

FIGURE 10.—Section of small hair follicle tumor (*right*) adjacent to a polyoma-virus-induced mammary tumor (*left*). Thin sheet of connective tissue separates the two neoplasms. Center of hair follicle tumor is necrotic. Hematoxylin and eosin. × 108



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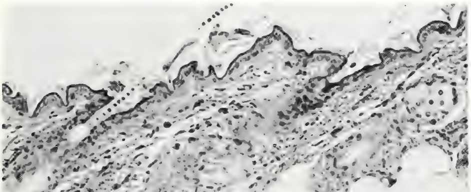

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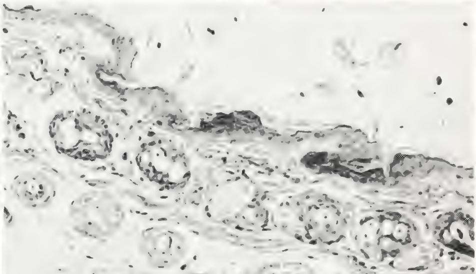

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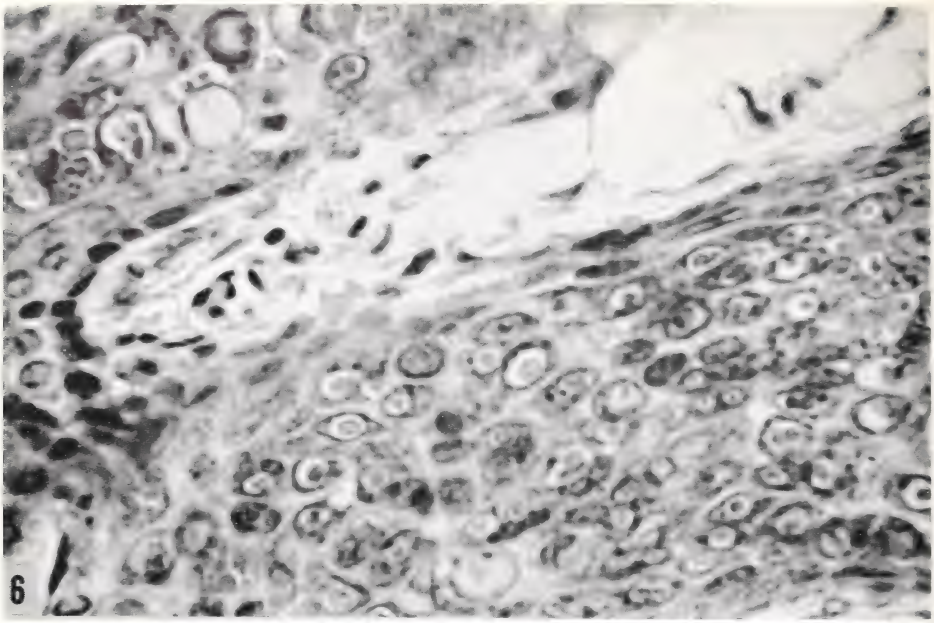
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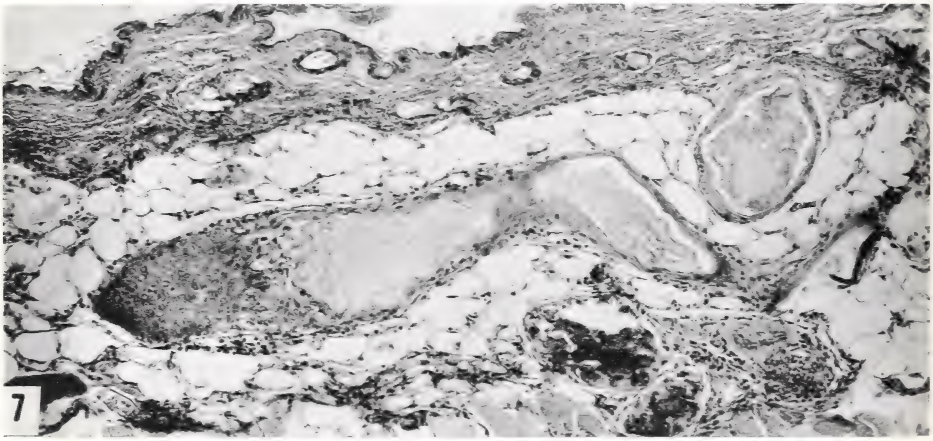
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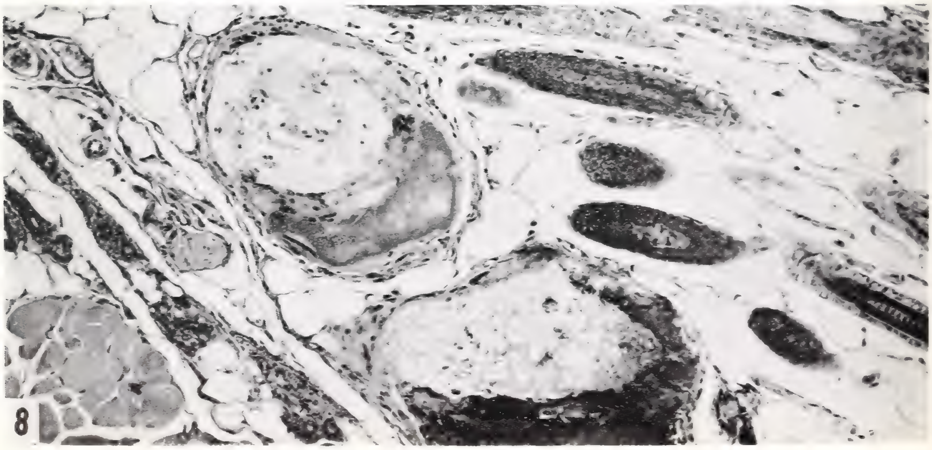
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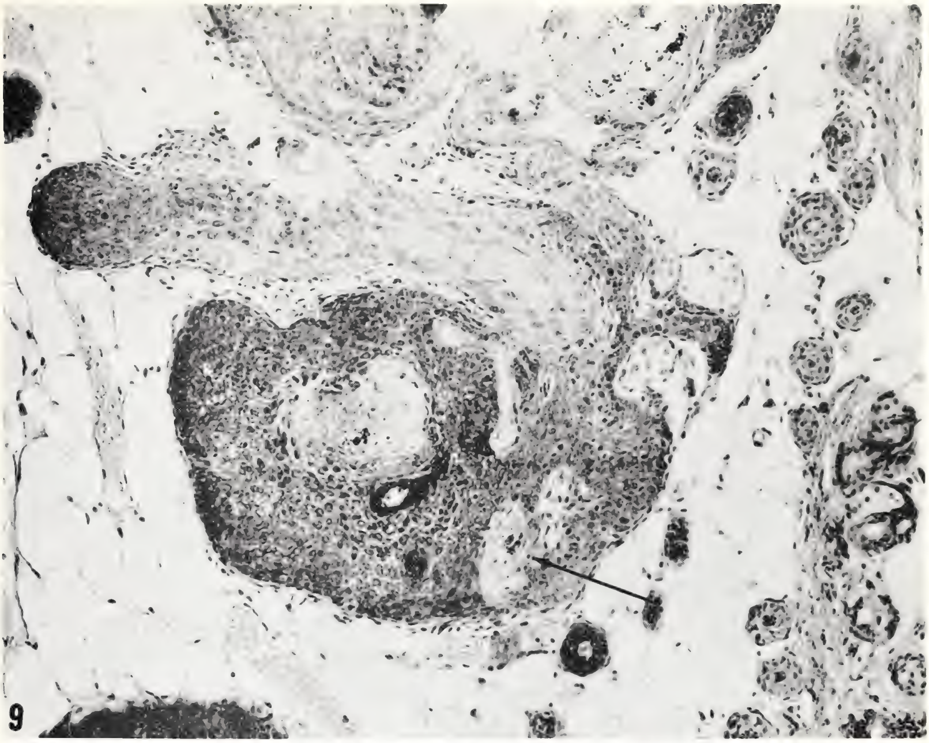

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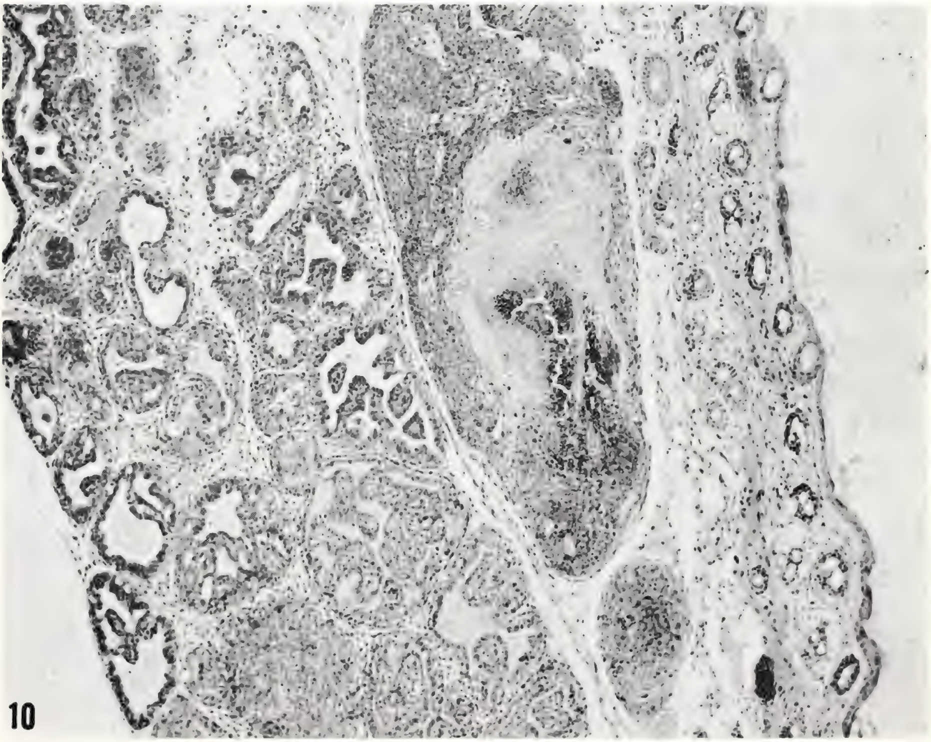

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PLATE 47

FIGURE 11.—Section through group of skin-appendage tumors, cut parallel to skin surface. Many lesions are completely necrotic. *Dark areas* represent viable cells. *Note* pale, round or elongated structures within many of necrotic lesions (*arrows*). These are keratinized bodies representing imperfect hair formation. Slightly darker material surrounding them is eosinophilic necrotic debris. Hematoxylin and eosin. × 19

FIGURE 12.—Von Kossa's stain of necrotic skin-appendage tumors, showing positive reaction for calcium (phosphates). *Note* "mummified epithelium" adjacent to black deposits representing calcification, on *left*. Granulomatous inflammatory reaction occupies areas between necrotic lesions. × 95

PLATE 48

FIGURE 13.—Section through several skin-appendage tumors of variable size, showing variable degrees of necrosis. Unaffected hair follicles overlying tumors are in resting phase of hair cycle. Hematoxylin and eosin. × 19

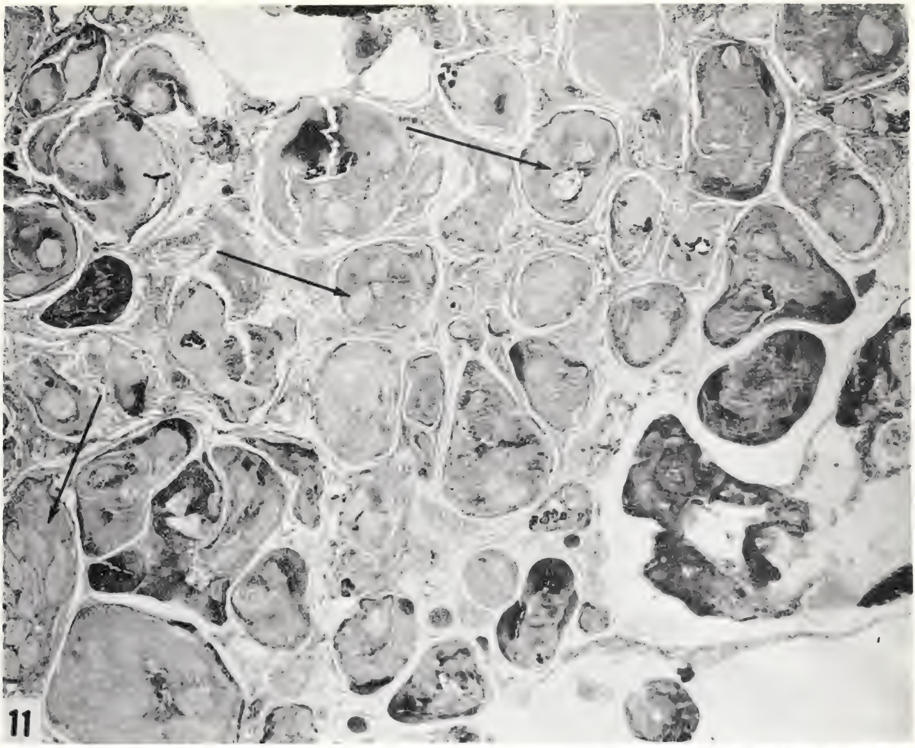
FIGURE 14.—Section through several skin-appendage tumors in area where hair follicles of overlying skin are in growth phase of hair cycle. Hematoxylin and eosin. × 19

FIGURE 15.—Section through skin from same block as that shown in figure 14. This section represents extension from right margin of section in figure 14. Here normal hair follicles are in resting phase, and appendageal tumors (*arrows*) are small. Hematoxylin and eosin. × 19

PLATE 49

FIGURE 16.—Pseudo-hair formation in skin-appendage tumor of an AKR mouse. Pale cylindrical structures made up of keratinized epithelium are formed by invaginations of the viable stratum of epithelium on periphery of tumor. Blood capillaries enter base of each pseudofollicle, simulating capillaries of dermal papillae. Hematoxylin and eosin. × 108

FIGURE 17.—Pseudo-hair formation in skin-appendage tumor of a C3H/Bi mouse. Here, as in tumors of other pigmented mice, dendritic melanoblasts accompany capillary vessels in centers of pseudofollicles. *Compare* with lack of pigment in pseudofollicles in figure 16. Hematoxylin and eosin. × 108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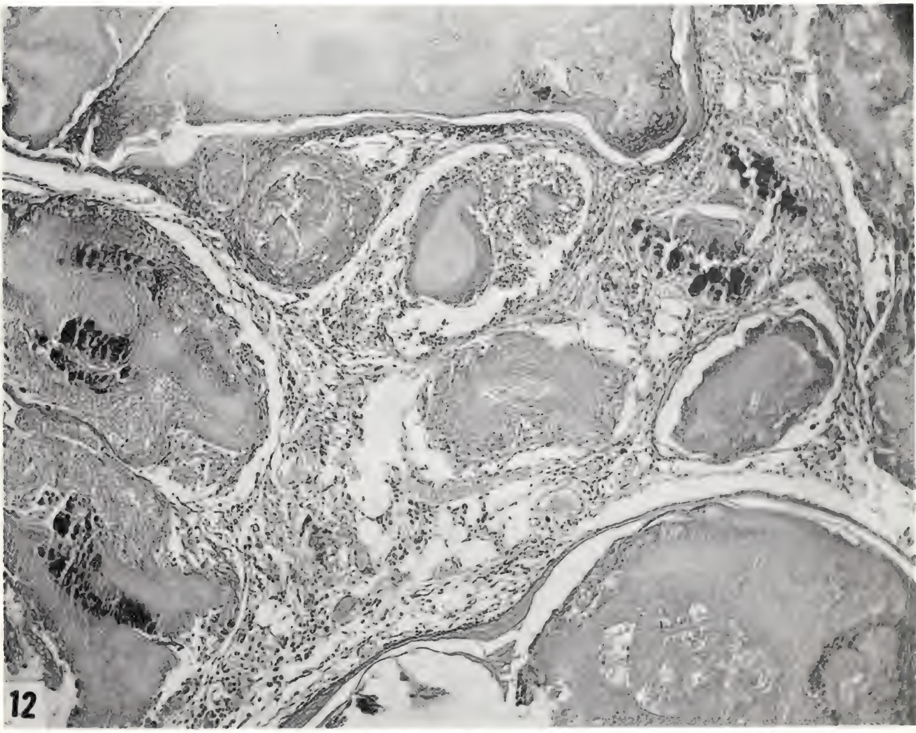
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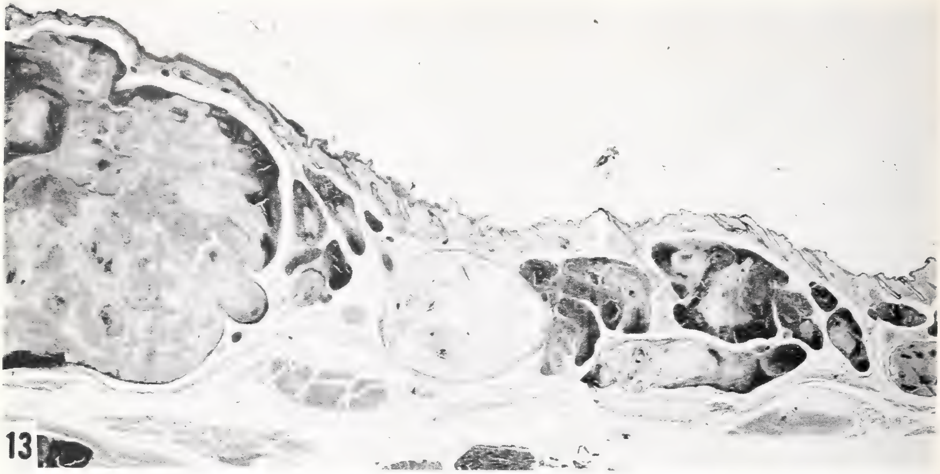

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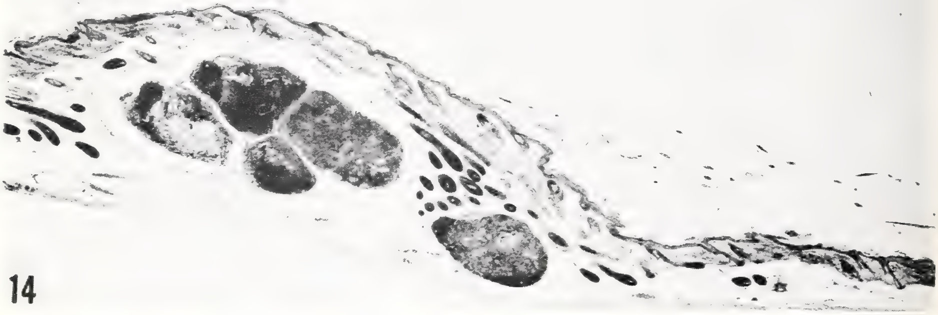

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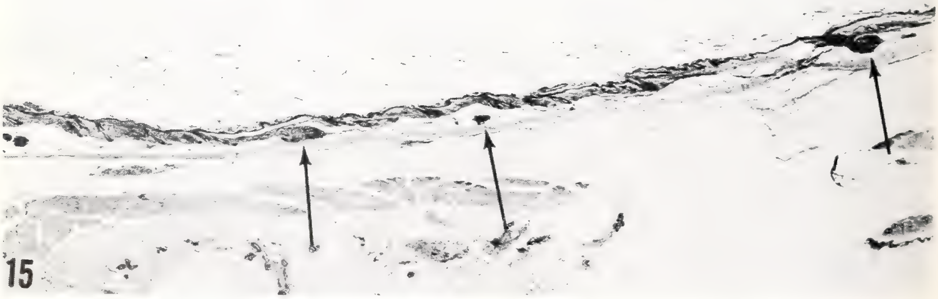

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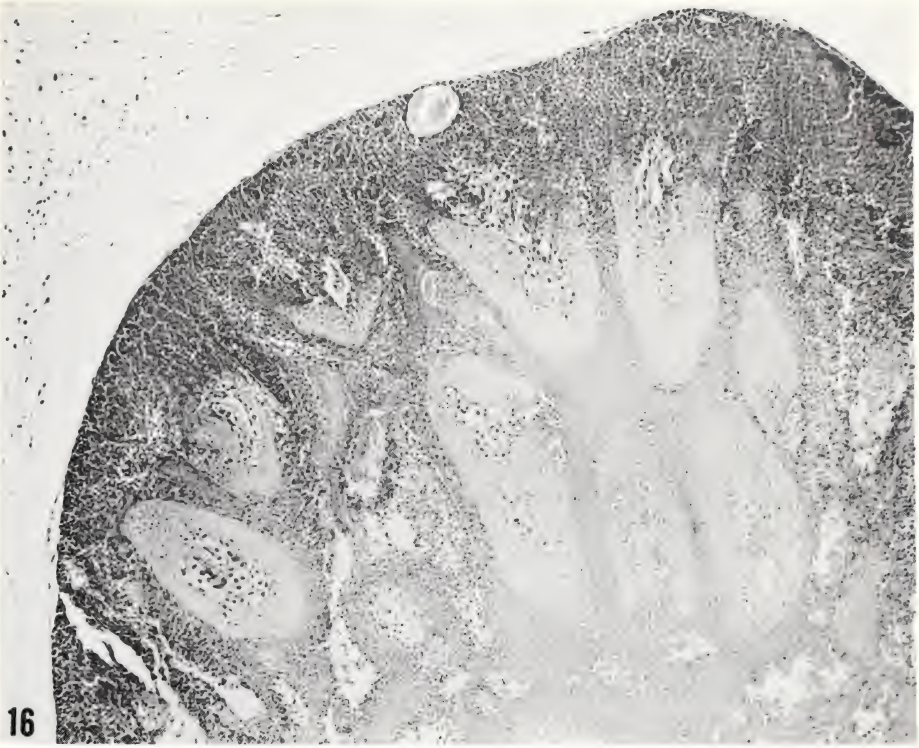

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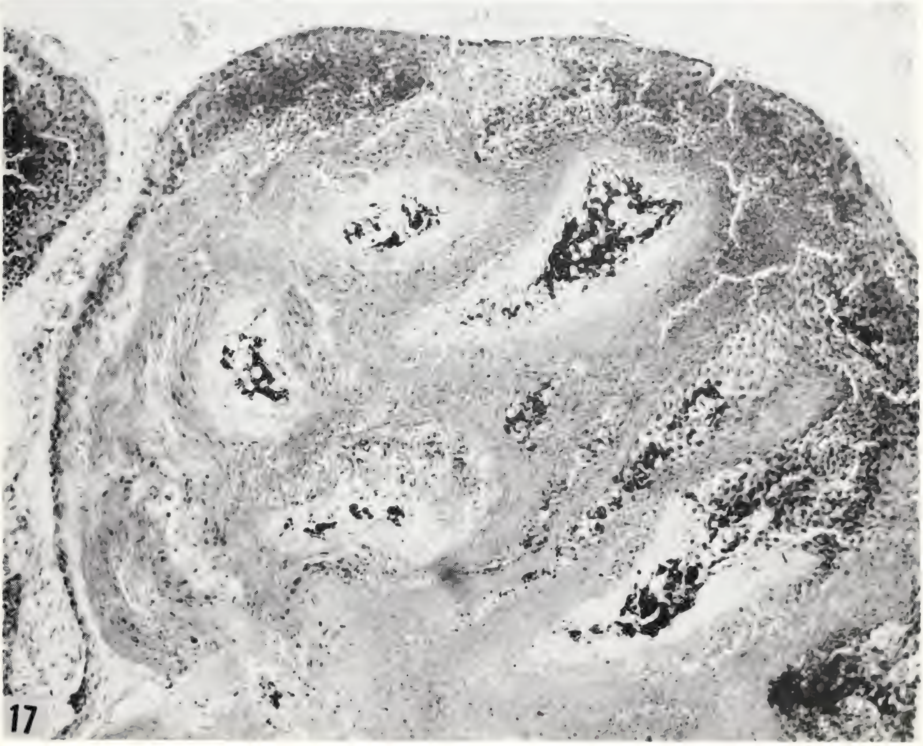

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PLATE 50

FIGURE 18.—Pseudofollicles in skin-appendage tumor of C3H/Bi mouse. *Note* central vascular core of each pseudofollicle. Keratinization resembles what normally occurs in cells of hair matrix. Pigment cells are conspicuous in keratinized cylinders. Hematoxylin and eosin. × 165

FIGURE 19.—Several pseudofollicles sectioned nearly transversely. Psuedofollicle at *far right* shows differentiation of internal and external hair sheaths. Capillaries surrounded by dendritic pigmented cells occupy *center* of pseudofollicle. Hematoxylin and eosin. × 165

PLATE 51

FIGURE 20.—Birefringence under polarized light demonstrates reaction consistent with keratinaceous composition of pseudohair in skin-appendage tumor. × 235

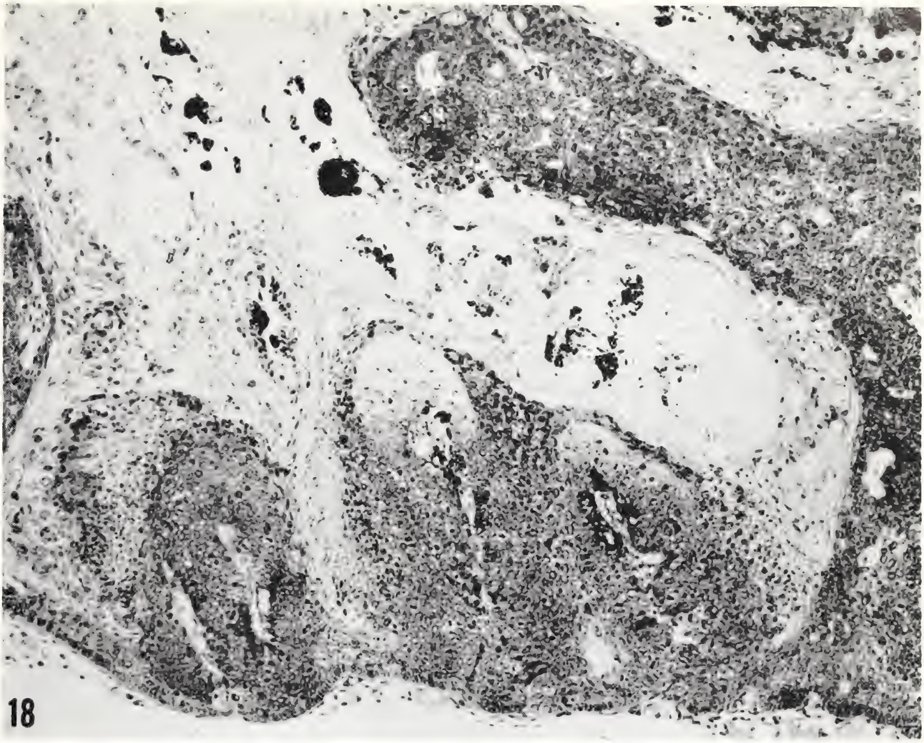
FIGURE 21.—Keratinizing cells of pseudohairs in skin-appendage tumor show acid-fast staining property similar to that of normal hair shafts (*left*). Ziehl-Neelson acid-fast stain. × 108

PLATE 52

FIGURE 22.—Section of skin-appendage tumor showing differentiation in 3 directions within single field. At *louer right* is pseudo-hair follicle. At *upper right* are small glandlike structures (*arrow*). At *upper left* and *center* are sebaceous cells. Hematoxylin and eosin. × 125

FIGURE 23.—Portion of skin-appendage tumor showing differentiation of sebaceous cells. *Note* mitotic figures in nonfunctioning cells from which sebum-producing cells appear to be stemming. Hematoxylin and eosin. × 235

FIGURE 24.—Portion of skin-appendage tumor showing formation of large, fluid-containing, glandlike spaces. *Note* that glandlike spaces are lined by double rows of epithelium. Hematoxylin and eosin. × 108



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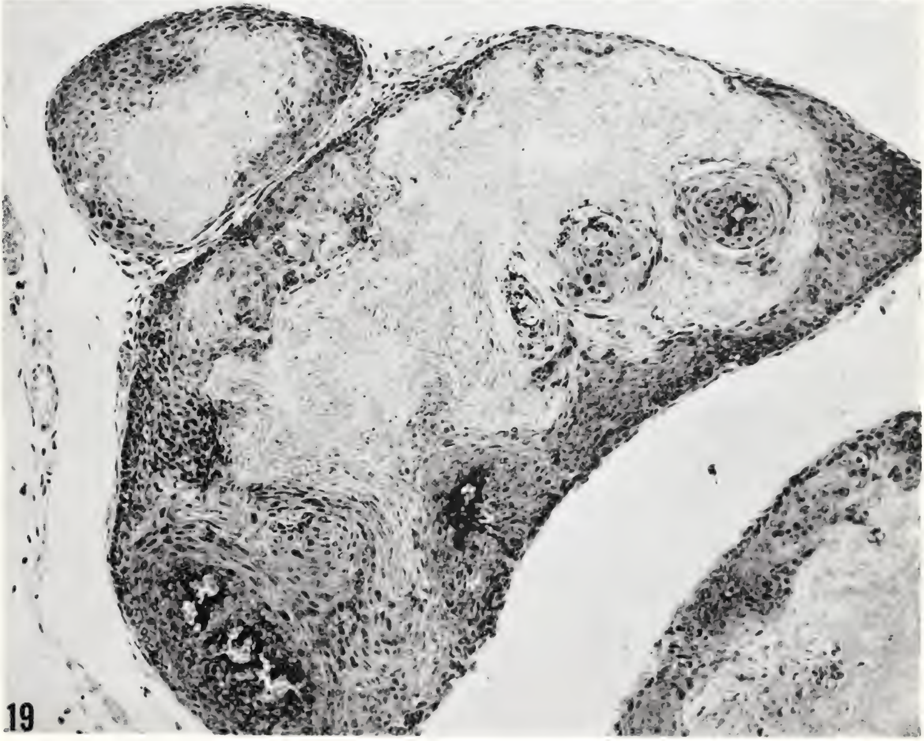

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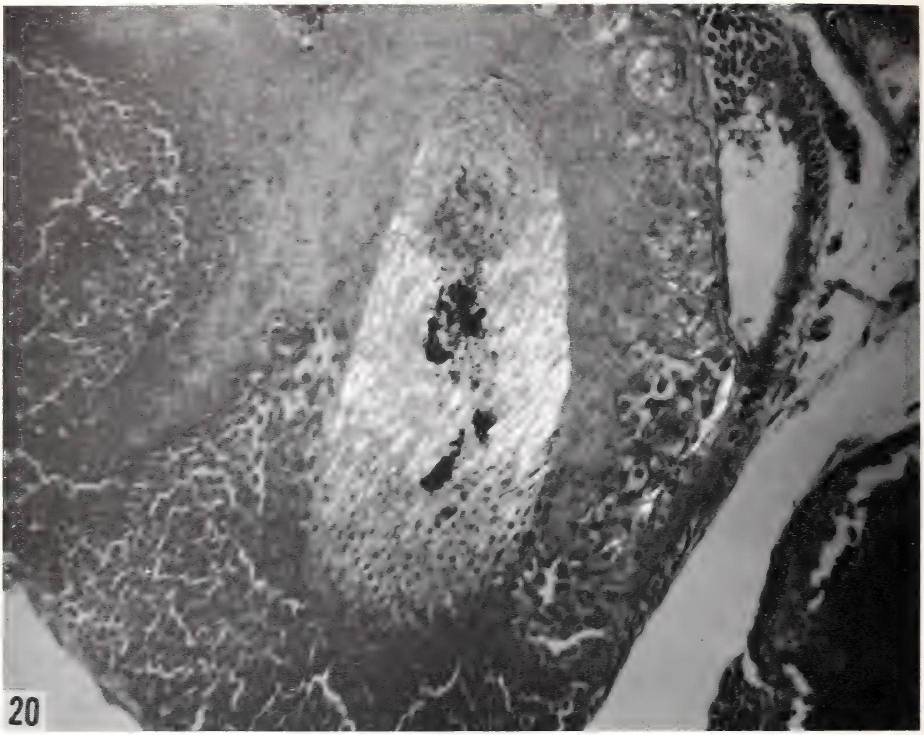

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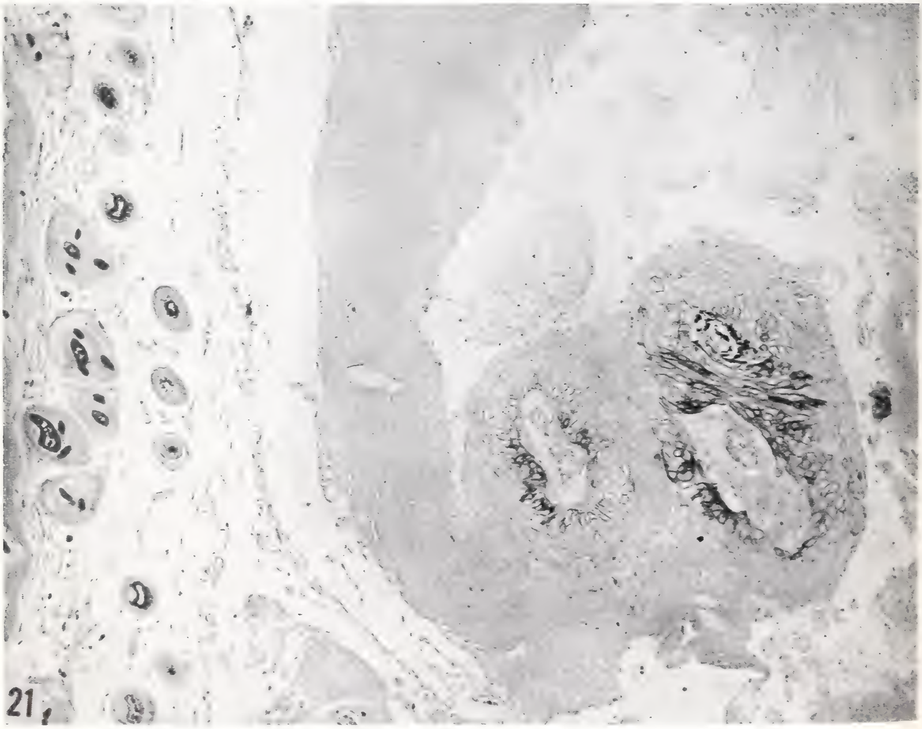

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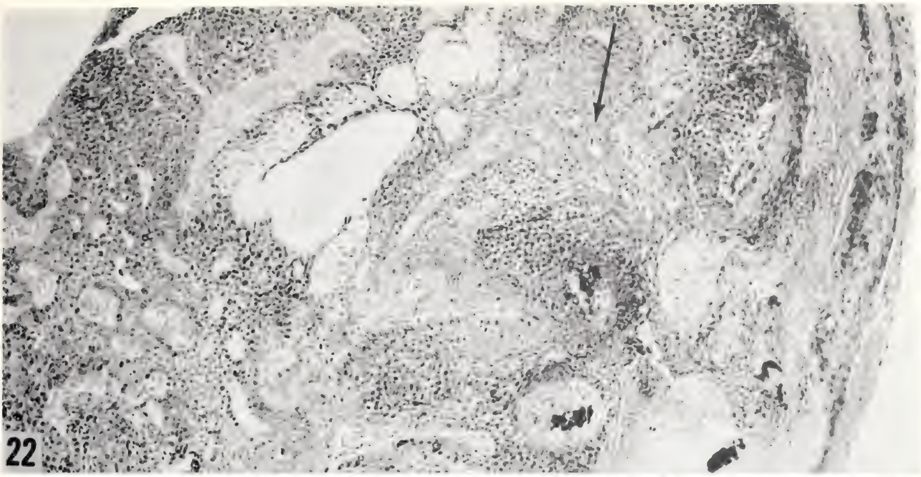

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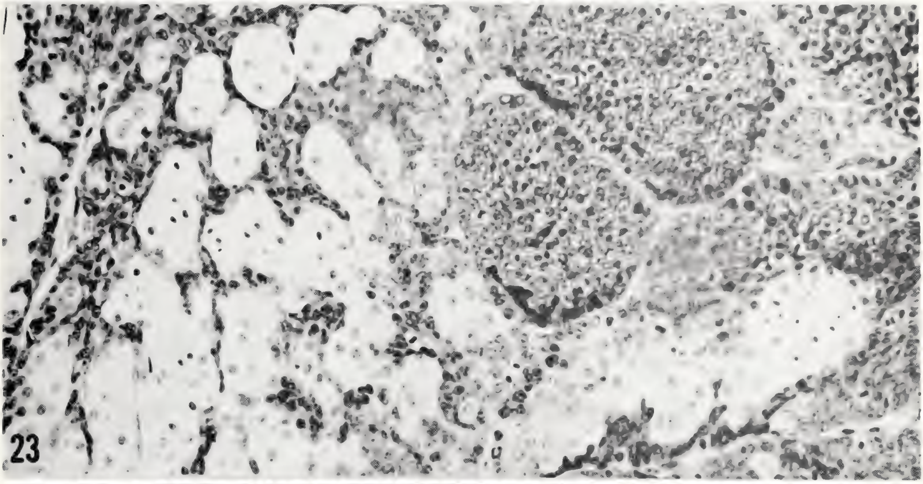

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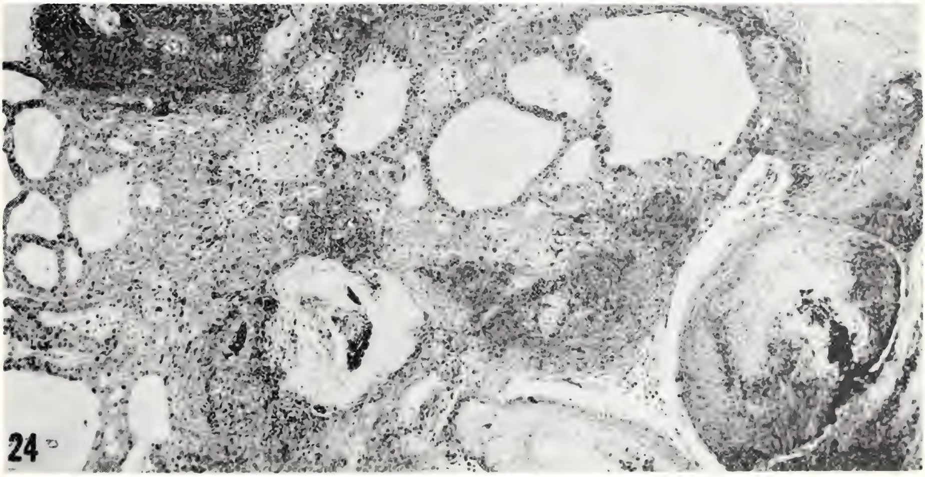


Plate 53

Figure 25.—Section of large skin-appendage tumors of mouse, at low magnification to show general pattern of central necrotic masses incompletely bounded by folded strata of viable epithelium. *Compare* with section of benign calcifying epithelioma of man, figure 26. Hematoxylin and eosin. × 19

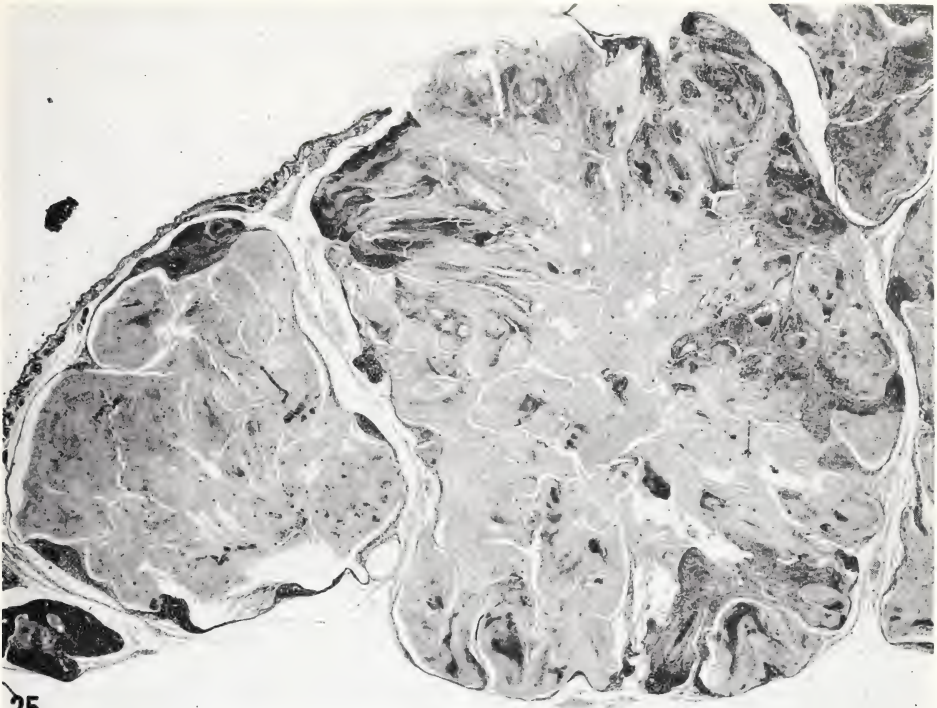
Figure 26.—Section of benign calcifying epithelioma of man (pilomatrixoma). Derivation of folds and sleeves of necrotic, partially keratinized epithelium from strata of viable epithelium (*dark areas*) is evident. Hematoxylin and eosin. × 19

Plate 54

Figure 27.—Section of calcifying epithelioma of man to show longitudinal and transverse sections of structures resembling the pseudohairs seen in skin-appendage tumors of mice. In this field, all epithelium is made up of dead "shadow cells." A vessel is present in *center* of transversely cut cylinder. *Compare* with figures 16 and 21. Hematoxylin and eosin. × 108

Figure 28.—A follicle-like structure in benign calcifying epithelioma, stained with Glenner and Lillie's method for protein-bound tyrosine. Dead "shadow cells" stain darkly, while viable epithelium gives little reaction. "Shadow cells" differ from those in skin-appendage tumors of mice (*see* fig. 29). × 95

Figure 29.—Portion of skin-appendage tumor of mouse, stained with Glenner and Lillie's method for protein-bound tyrosine. Like keratin of normal hair shaft at *top* of photo, shadow cells of this tumor react weakly or not at all. × 135



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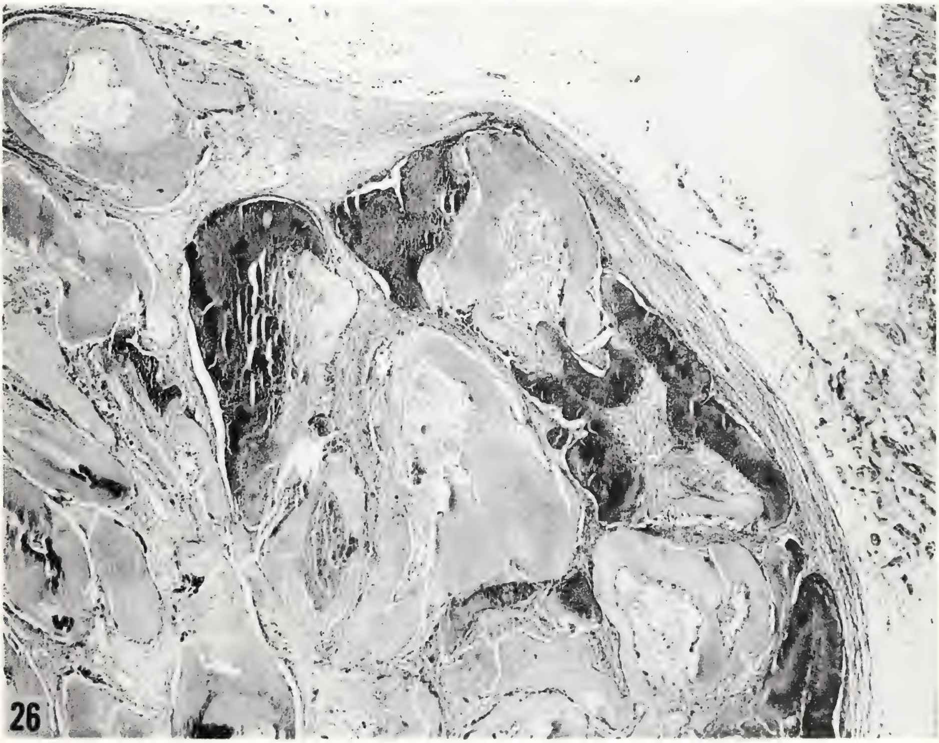

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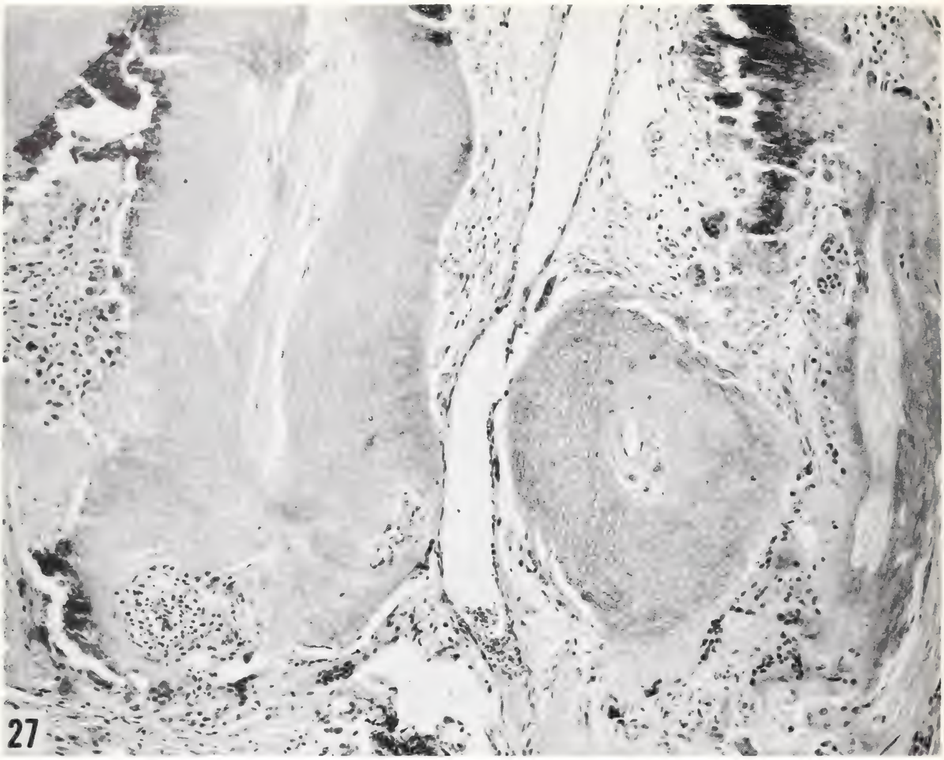
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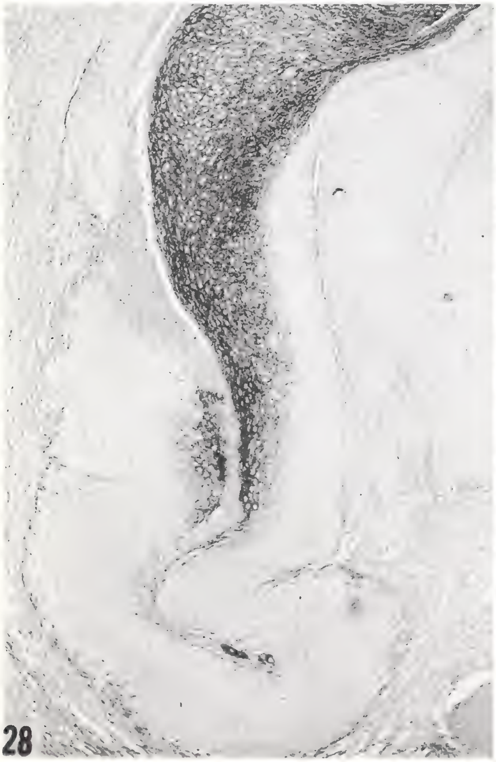
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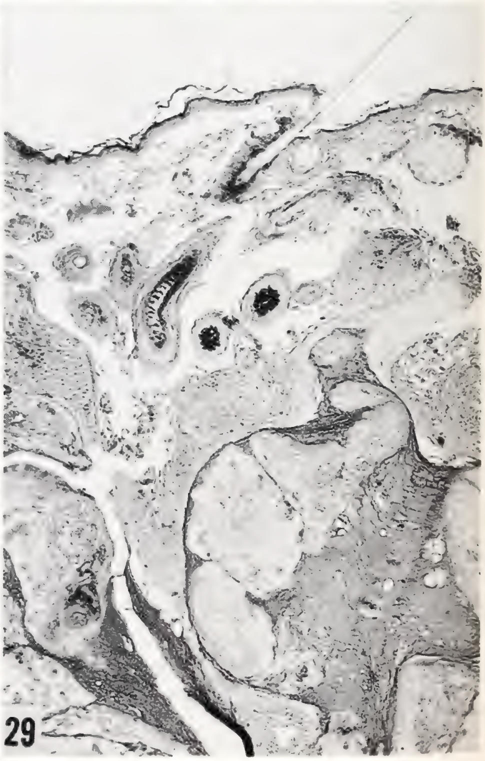
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DAWE

STUDIES ON PROMOTING ACTION IN SKIN CARCINOGENESIS[1, 2]

UMBERTO SAFFIOTTI and PHILIPPE SHUBIK,[3] Division of Oncology, Institute of Medical Research, The Chicago Medical School, Chicago, Illinois

SUMMARY

Experiments on two-stage skin carcinogenesis are described. They include tests for promoting action in mice of silver nitrate, iodoacetic acid, fumaric acid, ethylphenylpropiolate, emodin, oleic alcohol, monostearine, Span 20. These tests were essentially negative, though marked epidermal hyperplasia was often induced. *n*-Dodecane showed moderate activity in promoting tests. Certain petroleum fractions showed marked activity in promoting tests; some of these samples were also tested on rabbits. Studies on croton oil in mouse skin carcinogenesis are reported. A fraction of croton oil showed a promoting activity qualitatively similar to that of the original oil. Further fractionation and chemical studies of croton oil are in progress. The general significance of tests for promoting action is discussed.—NCI Monogr. No. 10: 489–507, 1963.

STUDIES OF the two-stage mechanism of skin carcinogenesis have been part of a long-term program of research undertaken in this laboratory during the past 12 years. When this program was begun, croton oil was known as the most effective "promoting agent." One problem facing us was the discovery of other promoting agents, and a variety of materials have been studied. During this period various substances with promoting action have been described by several groups of workers. In the present paper we will describe different experiments so far unpublished that have been undertaken during the past 10 years, several of which were begun before the demonstration of some of the newer promoting agents. We hope that bringing this material together in this way may help in the discussion of the general nature of promotion.

We have tested various materials for promoting activity on the skin of the mouse; some of these agents are pure chemical substances that have been selected for one reason or another, often because of their ability to

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

[2] Supported in part by grant CS-9212 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] Drs. A. C. Ritchie, G. Della Porta, and L. Tomatis contributed pathological study to some of the experiments. Mr. R. Feldman and Miss K. Spencer gave technical assistance.

induce hyperplasia and at other times because of reports that they had some action in carcinogenesis. Other mixtures have also been investigated, in particular certain fractions of catalytically cracked petroleum residues. Finally we have attempted to isolate the active material from croton oil in pure chemical form, and the progress of this fractionation study will be described.

TEST METHODS

All mouse tests were carried out in Swiss mice of the colony of this laboratory. This colony originated from Swiss mice obtained in 1951 from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and has since been bred randomly in this laboratory. The mice were kept in plastic cages with wood shavings and fed Rockland mouse diet and tap water *ad libitum*. A standard technique was used in all the tests with the exceptions that will be described. All substances were applied with a glass dropper on an area of skin approximately 2 × 2 cm in the interscapular region that had been clipped free of hair before the first application and subsequently kept free of hair by periodic clipping with scissors. The agents to be tested for carcinogenicity were applied twice weekly for the duration of the experiment. The agents to be tested for promoting activity were applied on mouse skin prepared by a single application of 7,12-dimethylbenz[*a*]anthracene (DMBA; Eastman Organic Chemicals) usually as a 1 or a 1.5 percent solution in mineral oil (Superla 34, Standard Oil of Indiana); the first application of promoting agent was given 1 week after the single application of carcinogen and thereafter the promoting agent was applied twice a week for the duration of the experiment. All animals were checked twice weekly, and skin lesions were charted weekly on graph paper (*1*). All mice were observed until spontaneous death or killed when dying. All tumor-bearing mice and many apparently tumor-free, with the exception of some that were lost through cannibalism, were examined histologically. Experimental treatments and results are reported in the tables that record the total number of skin-tumor-bearing animals and the total number of tumors observed. Skin lesions that persisted less than 4 weeks were not counted as tumors. Some tumors were found to be malignant either grossly or microscopically, and they are listed as carcinomas. The other tumors were all papillomas of the various types previously described (*1, 2*). Some papillomas were observed grossly and found to regress and are listed as regressions. The average latent period for the appearance of all the tumors is also recorded.

Different control groups of mice from the same colony were kept untreated and observed for their lifespan. In 240 females, 1 papilloma that regressed and in 240 males, 1 skin papilloma and a carcinoma of skin appendages were found. Two other groups of 100 males and 100 females were observed for over 100 weeks and showed no skin tumors. In other groups, painted with solvents only, an occasional tumor was found: 1 papilloma in 35 males and 1 carcinoma in 59 females painted with benzene 3 times per week, and 1 papilloma in 49 females painted with iso-octane 3 times per week. These results will be reported separately.

TESTS FOR PROMOTING ACTION

Experimental treatments and results are given in table 1. Groups with DMBA initiation and no further treatment were used as controls (expts. CH and DP–1).

Silver nitrate had been already tested (*3*) and found to be without promoting activity. The test was repeated for a longer period (expt. BQ-2). Silver nitrate produced marked epidermal hyperplasia; 3 mice of 20 developed 8 papillomas. Another group of mice (expt. BQ-1) was given also a single application of DMBA, but followed after 1 week by a single application of croton oil: One week later silver nitrate applications were begun for a test on the effect of an agent causing marked epidermal hyperplasia on a skin given initiation plus a single application of croton oil. Six mice of 20 bore 14 tumors. Recently Boutwell (*4*) reported that hyperplasia-inducing treatments, such as turpentine and wounding, are capable of carrying on the process of promotion when they are preceded by a limited course of croton oil treatment.

Iodoacetic acid, a potent SH reactor, was tested by Gwynn and Salaman (*5*) and reported to have promoting action. Its activity was retested in different conditions: In a first series of pilot tests a commercial sample of iodoacetic acid (Eastman Organic Chemicals) was used without further purification and given in a single application to 1 group (expt. AT-1), in 2 applications to another group (expt. AT-2), and once weekly to a third group (expt. AT-3), with negative results in the first 2 groups and few tumors in the third. Because of the relative instability of iodoacetic acid and the importance of ruling out the effect of possible impurities, a fourth group (expt. BI) was given twice weekly applications of a sample of iodoacetic acid that had been purified by repeated crystallizations and then kept in the dark under vacuum.[4] There was marked hyperplasia of the epidermis but no tumors were induced.

Fumaric acid, a possible breakdown product of iodoacetic acid, was also tested (expt. BO) and found to induce moderate focal hyperplasia of the epidermis but no tumors.

Ethylphenylpropiolate was suggested for testing because of its affinity for -SH groups, similar to that of iodoacetic acid.[5] A promoting test (expt. BA-1) and a carcinogenicity test (expt. BA-2) were carried out. No tumors were observed, but an intense hyperplasia of the epidermis was induced and maintained throughout the experiment in both groups of mice, though there was very little gross damage to the skin.

Emodin (trioxymethylanthraquinone), the active principle of the anthracene group of purgatives, was supplied by the S. B. Penick Co.,

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[4] Dr. R. Schoental suggested the purification and prepared the sample.

[5] Dr. F. L. Rose, F.R.S., Imperial Chemical Industries, Ltd., suggested the test and provided the sample.

TABLE 1.—Tests for promoting action in mice

Date experiment began	Experiment No.	Initiating treatment (once)*	Secondary treatment†	Initial No. of mice	Survivors (wks)		Duration of test (wks)	Total No. of tumor-bearing animals	Total No. of tumors	Total No. of regressions	Total No. of carcinomas	Average latent period (wks)
					10	20						
8/1953	CH	DMBA, 1% mineral oil	—	50 F	50	50	140	—	—	—	—	—
8/1955	DP-1	DMBA, 1% mineral oil	—	50 M	44	37	66	1	5	—	—	22
4/1953	BQ-2	DMBA, 1.5% mineral oil	Silver nitrate, 10% in water, twice weekly	20 M	18	13	44	3	8	—	—	19
4/1953	BQ-1	DMBA, 1.5% mineral oil	Croton oil, ‡ 5% in mineral oil, once; silver nitrate 10% in water, twice weekly	20 M	19	15	44	6	14	—	1	21
9/1952	AT-1	DMBA, 1.5% mineral oil	Iodoacetic acid, commercial, 0.9% in acetone, once	10 F	10	8	50	1	1	1	—	12
9/1952	AT-2	DMBA, 1.5% mineral oil	Iodoacetic acid, commercial, 0.9% in acetone, twice	10 F	10	10	50	—	—	—	—	—
9/1952	AT-3	DMBA, 1.5% mineral oil	Iodoacetic acid, commercial, 0.9% in acetone, once weekly	10 F	10	10	50	4	5	—	1	40
2/1953	BI	DMBA, 1% mineral oil	Iodoacetic acid, recrystallized, 0.9% in acetone, twice weekly	16 F	16	14	56	—	—	—	—	—
4/1953	BO	DMBA, 1.5% mineral oil	Fumaric acid, 1% in acetone, twice weekly	20 F	18	18	76	—	—	—	—	—
1/1953	BA-1	DMBA, 1% mineral oil	Ethylphenylpropiolate, 5% in acetone, twice weekly	10 F	10	10	80	—	—	—	—	—
1/1953	BA-2	—	Ethylphenylpropiolate, 5% in acetone twice weekly	10 F	10	9	91	—	—	—	—	—
11/1953	CK-2	DMBA, 1% mineral oil	Emodin, 0.5% in acetone, twice weekly	25 F	25	25	85	—	—	—	—	—
11/1953	CK-1	—	Emodin, 0.5% in acetone, twice weekly	25 F	25	21	85	—	—	—	—	—
3/1959	KZ-1, 2, 3	DMBA, 20 μg in 50 μl acetone, once	Oleic alcohol, twice weekly	30 F	30	30	82	3	3	1	1	34
3/1959	KZ-4, 5, 6	—	Oleic alcohol, twice weekly	30 F	30	30	82	2	2	—	—	58
6/1959	LH-3, 4	DMBA, 20 μg in 50 μl acetone, once	Monostearine, 5% in acetone, 3 times weekly	20 F	20	19	82	—	—	—	—	—
6/1959	LH-1, 2	—	Monostearine, 5% in acetone, 3 times weekly	20 F	17	17	82	—	—	—	—	—
1/1955	DQ-2	DMBA, 1% mineral oil	Sorbitan monostearate, twice weekly	50 M	41	26	75	5	8	2	1	25
1/1955	DQ-1	—	Sorbitan monostearate, twice weekly	50 M	43	22	73	1	1	—	—	30
5/1954	DH-1a	DMBA, 1% mineral oil	*n*-Dodecane, twice weekly	15 M	15	12	63	6	12	2	—	37
5/1954	DH-1b	DMBA, 1% mineral oil	*n*-Dodecane, twice weekly	15 F	14	14	63	6	14	5	2	41
5/1954	DH-2a	—	*n*-Dodecane, twice weekly	15 M	12	8	39	—	—	—	—	—
5/1954	DH-2b	—	*n*-Dodecane, twice weekly	15 F	15	15	63	2	2	2	—	40
7/1956	FB-1	—	*n*-Dodecane, 6 times weekly	30 F	30	29	52	—	—	—	—	—

*DMBA = 7,12-dimethylbenz[*a*]anthracene; mineral oil=Superla 34, Standard Oil of Indiana.

†Starting 1 week after initiation.

‡Croton oil (Boots, Nottingham, England).

New York, N.Y., and tested for promoting activity (expt. CK-2) and for straight carcinogenicity (expt. CK-1). No tumors were induced, and the epidermis was rendered only slightly hyperplastic.

Oleic alcohol (K & K Laboratories, Inc., Long Island City, N.Y.) was tested undiluted for carcinogenicity (expt. KZ-4, 5, 6) and for promoting action (expt. KZ-1, 2, 3) with essentially negative results and slight epidermal hyperplasia.

Monostearine (technical, Eastman Organic Chemicals) was tested for carcinogenicity (expt. LH-1, 2) and for promoting action (expt. LH-3, 4) and was found negative, with slight epidermal hyperplasia.

Sorbitan monolaurate (Span 20, Atlas Powder Co.) was tested following the experiments of Setälä and co-workers, reporting the promoting activity of this and related compounds (*6*). Span 20 was tested undiluted, with the same procedure used in this laboratory for other tests, for promoting action (expt. DQ-2) and for carcinogenicity (expt. DQ-1). No carcinogenic effect was detected, and minimal results were obtained in the test for promoting activity. Since the tests reported by Setälä and co-workers in their earlier papers were mostly performed by daily or even twice daily applications of the promoting agent, the discrepancy in the results could be explained by assuming that these substances need to be applied in much larger doses and much more frequently than promoting agents such as croton oil. This was subsequently found to be true (*7–10*).

n-Dodecane (Eastman Organic Chemicals) was tested undiluted for promoting action (expt. DH–1) and for carcinogenicity (expts. DH–2 and FB). No carcinogenic activity was detected even with the higher dose, but in the promoting test 12 mice of 30 bore 26 tumors. *n*-Dodecane also produced marked epidermal hyperplasia. Part of these results were previously reported (*11, 12*). An extensive investigation was set up for the study of the promoting activity of a series of normal paraffins and derivatives (*12*) and gave evidence that some of these aliphatic compounds show some activity in promoting tests, though very weak, so that large doses (amounting to gram quantities) are needed before the appearance of tumors.[6]

Other tests for promoting action performed in similar conditions in this laboratory have been previously reported. They include an extensive reinvestigation of polyoxyethylene sorbitan monostearate (Tween 60) (*2, 9, 10, 13*), which showed that the behavior of skin tumors, produced under promoting treatment with Tween 60, is remarkably similar to that previously described as characteristic of promoting agents in croton oil experiments (*14, 15*) in that they are mostly benign papillomas, many of which regress, while only a few carcinomas are found. Alkylbenzenes were investigated (*16*): A pilot-plant sample was found weakly active in a carcinogenesis test and markedly active in a promoting test. Extensive tests were then carried out on purified samples of the same alkylbenzene and on a series of commerical alkylbenzenes, which were all found negative in carcinogenesis tests but weakly active in promoting tests. A sample of creosote oil was studied chemically and submitted to biological testing (*17*): The results indicated a marked carcinogenicity of the sample, which was found to give a biological response similar to that of a carcinogenic polynuclear hydrocarbon in straight tests and after initiation with DMBA, in that the tumors induced in both types of tests included a high percentage of malignancies and no regressions. The basic fraction was inactive.

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[6] Studies on the promoting action of normal paraffins are continued and expanded in the Department of Pharmacology, The Chicago Medical School, by Dr. J. Sicé (personal communication), who, using purified samples of these compounds, confirmed the activity.

STUDIES ON PETROLEUM FRACTIONS

As the testing of new materials for carcinogenic and promoting activity was in part motivated in this laboratory by our interest in detecting environmental cancer hazards, we became greatly interested in a series of reports, appearing in 1951, on the carcinogenicity of petroleum products (*18*). Another reason for our interest in this report was that it showed that certain fractions of a catalytically cracked petroleum oil (boiling range 700–800° F) produced only papillomas in mice, while higher boiling fractions produced also carcinomas and lower boiling fractions were inactive: We tested these fractions [7] for promoting activity to explore the possibility of isolating a promoting agent from them with a fractionation procedure that might have proved easier than that for croton oil. The results of the first series of these tests have been previously reported (*19*, *20*) and are summarized in table 2 with the addition of survival rates and the average latent period for all the tumors (expts. CE, CD, and CC). The additional test of the fraction boiling between 600 and 650° F (expt. CU) showed also no activity in a carcinogenesis test, but still a considerable activity in a promoting test. A repetition of the tests of the lower boiling fractions several months later (expt. DF) showed again no carcinogenicity, but a marked reduction of the "promoting" activity. A third series of tests of the same samples, after a further 6 months, showed completely negative results (expt. DFR). The samples had been stored in metal cans at 4° C. In the meantime, a fractionation procedure was devised and several fractions were tested for promoting action. The results were mostly negative. The chemical studies aimed at isolating the promoting agents in these oil fractions were therefore discontinued especially in view of the loss of activity of the original samples.

Another test can be reported in this context. A commercial sample of a motor oil (Permalube, Standard Oil of Indiana) was found to be without any carcinogenic or promoting activity (expt. CV).

The fractions of a catalytically cracked oil that were tested on mice, as well as fractions of a steam-cracked petroleum tar, were tested on the skin of rabbits. Adult female New Zealand albino rabbits, obtained from

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[7] The samples were supplied by the Esso Laboratories, Standard Oil Development Co., now Esso Research and Engineering Co., Linden, N.J.

TABLE 2.—Tests of petroleum fractions in mice

Date experiment began	Experiment No.	Initiating treatment (once)*	Secondary treatment†	Initial No. of mice	Survivors (wks)		Duration of test (wks)	Total No. of tumor-bearing animals	Total No. of tumors	Total No. of regressions	Total No. of carcinomas	Average latent period (wks)
					10	20						
7/1953	CE–1	DMBA	MH–407‡, twice weekly	20 F	16	5	25	14	151	10	25	15
7/1953	CE–2	—	MH–407‡, twice weekly	20 F	15	6	25	7	45	1	21	17
7/1953	CD–1	DMBA	MH–406‡, twice weekly	20 M	18	8	25	13	191	23	4	13
7/1953	CD–2	—	MH–406‡, twice weekly	20 M	15	8	25	5	12	1	—	18
7/1953	CC–1	DMBA	MH–405‡, twice weekly	10 M	9	3	25	9	62	7	2	14
8/1953	CC–2	—	MH–405‡, twice weekly	20 M	19	8	25	—	—	—	—	—
2/1954	CU–3	DMBA	MH–404§, twice weekly	30 F	29	25	48	9	41	7	9	25
2/1954	CU–2	—	MH–404§, twice weekly	20 F	19	15	62	—	—	—	—	—
5/1954	DF–405–1	DMBA	MH–405§, twice weekly	30 M,F	29	20	40	3	5	—	—	27
5/1954	DF–405–2	—	MH–405§, twice weekly	30 M,F	26	13	40	—	—	—	—	—
5/1954	DF–404–1	DMBA	MH–404§, twice weekly	30 M,F	28	22	40	4	7	—	3	22
5/1954	DF–404–2	—	MH–404§, twice weekly	30 M,F	29	25	40	—	—	—	—	—
11/1954	DFR–405–1	DMBA	MH–405 50% in mineral oil, twice weekly	30 M,F	14	12	44	—	—	—	—	—
11/1954	DFR–405–2	—	MH–405 50% in mineral oil, twice weekly	30 M,F	20	16	44	—	—	—	—	—
11/1954	DFR–404–1	DMBA	MH–404 50% in mineral oil, twice weekly	30 M,F	17	14	44	—	—	—	—	—
11/1954	DFR–404–2	—	MH–404 50% in mineral oil, twice weekly	30 M,F	23	20	44	—	—	—	—	—
1/1954	CV–1	DMBA	Permalube motor oil, twice weekly	14 F	12	10	66	—	—	—	—	—
1/1954	CV–1	—	Permalube motor oil, twice weekly	18 F	18	18	66	—	—	—	—	—

*7,12-Dimethylbenz[*a*]anthracene (DMBA), 1 percent in mineral oil (Superla 34), Standard Oil of Indiana.

†Starting 1 week after initiation. Samples: fractions from a high boiling catalytically cracked oil filtered free of sediment (MH-191), with the following boiling ranges: MH-407 = 750 to 800° F; MH-406 = 700 to 750° F; MH-405 = 650 to 700° F; MH-404 = 600 to 650 °F (*18*).

‡Undiluted for 10 weeks, then 50 percent in acetone for 15 weeks. These tests were reported previously (*20*).

§Undiluted.

Abrams Small Stock Breeders, Chicago, Illinois, were used and maintained as previously reported *(20)*. Two different groups of tests were performed; the experimental treatments and the results are reported in table 3. The first group of tests (experimental series A) was previously reported *(20)* and showed that oil fractions, that when tested in mice were found inactive in carcinogenesis tests and only active in promoting tests, do possess carcinogenic activity for the skin of the rabbit. In a second group of tests (experimental series B), the experiments were performed on 8 rabbits. Each rabbit was painted once with DMBA, 1.5 percent in mineral oil on 4 areas (both ears and both flanks); beginning 1 week later, each of the 4 samples under test was then applied twice weekly on 2 rabbits by painting the 2 areas on the left side (ear and flank) in 1 rabbit and on the right side in the other rabbit, leaving the remaining 2 areas of each rabbit with just 1 application of DMBA and no further treatment. The results show a remarkably increased tumor incidence and a shorter latent period when the oil fractions were painted after initiation with DMBA as compared with straight tests. A few tumors were found on ears painted with DMBA once, but never on the flanks: These tumors were most likely due to contamination from the ear of the opposite side that was painted with the sample under test.

TABLE 3.—Tests of petroleum fractions on rabbits

Experimental series*	Initiating treatment†	Secondary treatment‡	Number of skin areas painted	Number of skin areas with tumors	Total No. of tumors	Average latent period (wks)
A	—	MH-404	6	3	12	24
A	—	MH-405	6	5	22	16
A	—	MH-406	6	4	29	14
A	—	MH-407	6	5	39	17
A	—	MH-192	6	1	8	22
B	DMBA	MH-192	4	4	43	19
B	DMBA	—	4	1	9	31
A	—	MH-393	6	2	5	28
B	DMBA	MH-393	4	4	14	23
B	DMBA	—	4	—	—	—
B	DMBA	MH-394	4	4	23	31
B	DMBA	—	4	2	2	33
B	DMBA	MH-395	4	4	32	25
B	DMBA	—	4	1	2	43

*The tests of series A were previously reported *(20)*.

†7,12-Dimethylbenz[*a*]anthracene (DMBA), 1.5 percent in mineral oil (Superla 34, Standard Oil of Indiana).

‡Oil fractions applied undiluted twice weekly starting 1 week after initiation. Samples: fractions from a high boiling catalytically cracked oil filtered free of sediment (MH-191), with the following boiling ranges: MH-407 = 750 to 800° F; MH-406 = 700 to 750° F; MH-405 = 650 to 700° F; MH-404 = 600 to 650° F; MH-192 = 0 to 700° F; fractions from a steam-cracked petroleum tar (MH-109), with the following boiling ranges: MH-393 = from the initial boiling point to 600° F; MH-394 = 600 to 650° F; MH-395 = 650 to 700° F *(18)*.

STUDIES ON CROTON OIL

The promoting action of croton oil has been studied now for over 20 years, since its first discovery by Berenblum in 1941 (*21*). Its mode of action in mouse skin carcinogenesis has been described by Berenblum and Shubik (*22–25*). Subsequent studies have shown that the biological response induced by croton oil after a single application of carcinogen is characterized by the fact that a high percentage of the induced tumors regress and very few become malignant, while a high percentage of malignancies and few, if any, regressions are produced by repeated applications of carcinogens (*14, 15*). A single application of carcinogen in a volatile solvent at appropriate dose levels without further treatment produced a high percentage of regressions but also a considerable proportion of malignancies (*26*). When croton oil was applied once, at the same time as the single application of carcinogen, no promoting effect was noted and the qualitative distribution of the tumors was not substantially changed (*27*). That croton oil could produce a few tumors by itself was suggested by several reports (*28–30*). Control of all the experimental conditions is necessary in studies of this type before assessing the significance of their results (*31*).

Examples of tests of croton oil alone are reported in table 4 (expts. BV-1, 2 and BW-1, 2). Mostly negative results with croton oil were also found in some experiments testing the initiating power of certain treatments, such as local anoxia (*32*), benzoyl peroxide (expt. CS-2), and di-*t*-butyl peroxide (expt. CT-2), which were also found negative in carcinogenesis tests (expts. CS-1 and CT-1). Similar negative results were obtained in tests using chloroacetophenone or dinitrophenol mixed with croton oil (expts. CQ and CR).

As opposed to the results obtained with croton oil alone or without any effective initiation, results obtained with croton oil applied to previously initiated mouse skin showed the typical promoting effect. Several such groups have been reported (*15, 32–35*). Croton oil was also tested on the skin of the hamster, a species otherwise susceptible to skin carcinogenesis, but no effect was observed (*36, 37*); negative results with Tween 60 were also obtained in hamsters (*37*). Berenblum has suggested since 1941 (*38*) that initiation of the skin of the mouse could be induced not only by topical applications but also by a remotely acting carcinogen. His test with intraperitoneal injections of benzo[*a*]pyrene combined with croton oil painting unfortunately did not show any effect in the experimental conditions used. The hypothesis was then tested again with the use of *N*-2-fluorenylacetamide (2-acetylaminofluorene) by oral administration followed by croton oil, and positive results were obtained (*35*). The mechanism of initiation by remotely applied carcinogens followed by croton oil was then demonstrated by several investigatiors with different carcinogens (*39–44*).

Croton oil is a complex mixture and has different pharmacological and toxicological properties: It is a cathartic, a topical irritant, a vesicant, and a tumor-promoting agent for the skin of the mouse. It was felt that only by isolating the tumor-promoting principle from croton oil could the biological nature of its promoting action be studied in detail. Two fractionation studies were undertaken in this laboratory. The first procedure was developed and described by Dr. J. Sicé (*45, 46*) and con-

TABLE 4.—Tests of croton oil in mice

Date experiment began	Experiment No.	Initiating treatment*	Secondary treatment†	Initial No. of mice	Survivors at weeks		Duration of test (wks)	Total No. of tumor-bearing animals	Total No. of tumors	Total No. of regressions	Total No. of carcinomas	Average latent period (wks)
					10	20						
8/1953	BV-1	—	Croton oil,‡ 1.5% in acetone, twice weekly	21 M	20	14	42	2	3	—	—	16
8/1953	BV-2	—	Croton oil,‡ 1.5% in benzene, twice weekly	21 M	21	13	42	3	4	1	—	25
8/1953	BW-1	—	Croton oil,‡ 1.5% in acetone, twice weekly	21 M	21	12	42	1	2	2	—	23
8/1953	BW-2	—	Croton oil,‡ 1.5% in benzene, twice weekly	21 M	15	13	42	3	3	—	—	31
12/1953	CS-2	Benzoyl peroxide, 0.5% in acetone, twice weekly for 3 weeks	Croton oil,‡ 5% in mineral oil,§ twice weekly	20 F	15	15	67	—	—	—	—	—
12/1953	CS-1	Benzoyl peroxide, 0.5% in acetone, twice weekly	—	21 F	18	17	80	—	—	—	—	—
12/1953	CT-2	Di-*t*-butyl peroxide, 0.5% in acetone, twice weekly for 3 weeks	Croton oil,‡ 5% in mineral oil,§ twice weekly	20 F	16	16	80	1	1	1	—	44
12/1953	CT-1	Di-*t*-butyl peroxide, 0.5% in acetone, twice weekly	—	20 F	18	16	81	—	—	—	—	—
12/1953	CQ	—	Chloroacetophenone, 1.5% in a 0.5% solution of croton oil‡ in acetone, twice weekly	30 F	28	26	52	1	1	—	—	46

12/1953	CR	—	Dinitrophenol, 1.0% in a 0.5% solution of croton oil‡ in acetone, twice weekly	30 F	17	16	76	—	—	—	—	—
6/1955	EA-1	DMBA,‖ 100 μg in 50 μl acetone, once, in resting phase	DJ-14,¶ 0.1% in acetone, twice weekly	22 F	22	21	70	20	126	70	9	21
6/1955	EA-2	DMBA,‖ 100 μg in 50 μl acetone, once, in growth phase	DJ-14,¶ 0.1% in acetone, twice weekly	22 F	19	18	70	18	119	52	9	17
6/1955	DZ-1	Urethan, 20 mg intraperitoneally daily for 5 days	DJ-14,¶ 0.1% in acetone, twice weekly	30 F	24	18	50	20	118	60	2	18

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*In experiments CS-1 and CT-1 the treatment was continued for the duration of the test.

†Starting 1 week after the end of the initiating treatment.

‡Croton oil was obtained from Boots, Nottingham, England, except for expt. BW, where it was from S. B. Penick & Co., New York, N.Y., kindly made available to us by Dr. R. K. Boutwell.

§Mineral oil = Superla 34, Standard Oil of Indiana.

‖DMBA = 7,12-dimethylbenz[*a*]anthracene.

¶DJ-14 = hexane-eluted fraction of a methanolic extract of croton seeds (*46*).

sisted of a differential solvent extraction of croton seeds and a subsequent chromatographic separation on silica gel. It led to the concentration of promoting activity in some fractions separated from the most vesicant fractions.

The hexane-eluted fraction of a methanolic extract of croton seeds, coded DJ-14 (*46*), was used in several biological tests, examples of which are given in table 4. Two groups of mice were separated according to their hair cycle by clipping the hair on the back and then observing whether it grew back during the next week. The mice with no hair growth were put in one group (expt. EA-1) and those with active hair growth in another (expt. EA-2). Both groups were then initiated with 100 μg of DMBA (50μl of a 0.2% solution in acetone), and 1 week later the promoting treatment with DJ-14 was started. No basic difference in tumor yield was seen in the 2 groups. In both groups the fraction DJ-14 produced a typical promoting action, and the biology of the induced tumors again showed a high percentage of regressions and a low incidence of malignancies. The significance of the hair cycle effect on mouse skin carcinogenesis has been extensively investigated by Berenblum, Haran-Ghera, and Trainin (*47*). The present results conform to their finding that, when croton oil is given, the total tumor yield is the same either with initiation applied in resting phase or in growth phase. Another experiment was done with the use of systemic initiation (expt. DZ-1). Five intraperitoneal injections, each of 20 mg of urethan in saline, on 5 consecutive days, were followed 1 week later by the promoting treatment with DJ-14. Again the biological effect of the promoting agent was similar to that of croton oil. The initiating action of intraperitoneal injections of urethan followed by croton oil was reported by Berenblum and Haran-Ghera (*41*) and by Ritchie (*43*).

The promoting fraction DJ-14 was also used in other tests (*48–50*). A dose-response study of promoting action was carried out with this fraction (*50*): Its results indicated that the dose of the promoting agent was directly related to the total incidence of tumor-bearing mice and inversely related to the length of the latent period, and suggested that the critical factor of the tumor response was the total cumulative dose of promoting agent.

DISCUSSION

When a discussion on carcinogenesis turns to the problems of a two-stage mechanism of tumor induction, often a considerable degree of confusion and lengthy controversies arise on the significance of the different findings offered by various investigators (*53–55*). A number of such arguments, sometimes carried through the published discussions of experimental papers, seem to come unstuck on semantic differences or on some too literal interpretations of theoretical views offered as working hypotheses. On the other hand, such discussions often deviate to encompass the whole field of carcinogenesis, thus extrapolating the results obtained on one tissue or one animal species to a number of other unrelated instances. Our knowledge of a two-stage mechanism of tumor induction is still essentially limited to skin carcinogenesis, and it is extremely difficult to extend its characteristics to other aspects of tumor pathogenesis.

It is not our purpose here to review once more the whole field of studies on promoting action, since extensive reviews on the subject are available (*56–58*) and our views have been expressed before (*9*, *10*, *13*, *15*, *59–61*). The experiments reported here will be used to discuss a few points.

The series of tests of different substances reported in table 1 confirms that the induction of a lasting hyperplasia of the epidermis is not a sufficient requirement for the induction of promoting action.

Certain substances have been reported in the literature as promoting agents with a low degree of activity; an example is *n*-dodecane, requiring a very large dose, amounting to gram quantities, to produce a noticeable promoting effect. It is difficult to assess the significance of the role of such agents in carcinogenesis, since it is hard to rule out the presence of contaminants in the test materials. For *n*-dodecane, as for Tween 60, the effective dose is several hundred times higher than that of the active croton oil fractions (*10*, *50*), and the mechanism of action might well be of different nature from that of croton oil, though the biological effects of the two different treatments show many close similarities.

The studies on petroleum fractions gave initially some hope for detecting a fraction with marked promoting activity that could be submitted to further chemical analysis, but unfortunately the results were largely negative and repetitions of the tests showed that the samples gradually lost their activity. The experience provided by these tests is a good example of some pitfalls in studies of carcinogenicity and promoting action. When tests are carried out with samples of unknown composition, care should be taken to control all possible variables, and the tests should be repeated after some time before any final conclusion is drawn. Tests for promoting action should be examined in detail and carefully evaluated in different experimental conditions: For example, the results of the tests in mice of fractions MH-405 and MH-404 (table 2, expts. CC and CU), taken out of context and with only an indication of the total number of tumors induced, could be easily brought forth as evidence of a genuine promoting action. But an analysis of the tumors induced shows that the percentage of regressions is considerably low, which is definitely at variance with the results obtained with croton oil and Tween 60; besides, fractions with boiling range just above those two show a full carcinogenic activity (table 2, expts. CD and CE). Tests in rabbits revealed carcinogenic activity for all these fractions (table 3). On the other hand, the fractions with weak or no carcinogenic activity in tests with mice show such a considerable activity after initiation that their effects seem far beyond a simple summatory action of two carcinogens which would only decrease the latent period and slightly increase the total yield of tumors (*15*, *17*, *62*). The results then are still compatible with the presence of a promoting factor, but of a rather weak one, possibly acting together with a weak carcinogenic factor.

And so we find ourselves back to considering croton oil. It is still the best promoting agent known in mouse skin carcinogenesis. Relatively small quantities are effective and give a qualitatively constant pattern of tumor response with different types of initiation and in different conditions. A "background carcinogenicity" for croton oil, that is, the appearance of a few tumors in animals treated with croton oil alone, has been reported and discussed several times (*63*). In some instances it seems to have been attributable to contamination of the animals with carcinogens before the applications of croton oil (*30, 31*). The tests reported here (table 4, expts. BV and BW) support this view. Still, there are sometimes a few tumors that appear in experiments with croton oil alone, which cannot be clearly explained. We know that a few occasional tumors can be found even in mice that are not given any treatment, as we reported at the beginning of this paper. If untreated or solvent-treated mice have a carcinogenic "potentiality" to develop an incidence up to 0.5 to 2 percent of skin tumors, it is not surprising that, when painted with croton oil, they may develop tumors in as many as 10 percent, and this does not necessarily demonstrate a definite carcinogenicity for croton oil, since we know it to be such a potent promoter.

The crux of the matter is that croton oil produces a large number of tumors in a short time when applied after initiation, and none or just a few if applied alone. No carcinogen applied after initiation has ever been shown to produce as many tumors and as quickly as croton oil at a dose that, applied without initiation, just produces few, if any, tumors. With repeated applications of carcinogens, all that a previous initiation does to the results is to shorten somewhat the latent period and increase slightly the total incidence (*15, 17, 62*) in a manner entirely compatible with the general pattern of summation effects of full carcinogens. With croton oil, a previous initiation changes the biological response entirely, with a striking increase in tumor incidence and a remarkably short latent period. In addition, there is the qualitative difference (many regressions, few malignancies) discussed before (*13, 15, 60*). These differences are so evident that certain misconceptions of this process are hardly understandable (*62, 64*). Tests of croton oil as well as Tween 60 on the skin of the hamster (*36, 37*), a species otherwise susceptible to skin carcinogenesis, gave entirely negative results and no background carcinogenicity was detected. It is certainly possible that the whole phenomenon of promotion has some species specificity as well as tissue specificity: If we consider it in its biological essence, as described previously, promorion is best represented by the effects of wound healing in rabbit ear carcinogenesis (*65*) and of croton oil in mouse skin carcinogenesis (*23*). It is our view that the term "promoting action" should be used with great caution in other instances, since its application to other examples of carcinogenesis may be misleading. In conclusion, we consider the "croton oil effect," as it may be called (*60*), a well-defined biological response quite different from that of known carcinogens. The other closest examples are the effects of Tweens and those of phenol (*66*).

One thing that became apparent from different studies is that the total dose of promoting agent given is directly correlated with the total yield of tumors and inversely with the latent period, as shown by studies with a croton oil fraction (*50*), Tween 60 (*8, 10*), and phenol (*66*).

The investigations on croton oil are now continued with fractionation studies. A fraction from the first fractionation procedure (*46*) was shown to act just as the original croton oil (table 4, expts. EA and DZ), though its activity was more concentrated and the sample less irritant. Further fractionation studies led to a concentration of the active material into 5 percent of the original oil (*51*). It is hoped that the chemical studies now under way (*52*) will lead to the identification of the tumor-promoting agent. It will then be possible to attack more closely the study of these effects at the level of cell physiology.

This will eventually clarify the nature of the croton oil effect and show how much it is related to the mode of action of full carcinogens. The role of croton oil was originally taken to represent a model for one of the two stages of action of carcinogens in general, namely promotion. The characteristics of the croton oil effect that have been subsequently described show that it is clearly distinct from the total or partial effect of a full carcinogen, actually so much distinct that it may be entirely unrelated to the mechanism of action of full carcinogens. The studies on croton oil have led to the identification of the immediate and lasting effect of a single application of carcinogen, known as initiation. Different factors can determine the subsequent course of skin carcinogenesis, following initiation: (*a*) full carcinogens, which will complete the carcinogenic process up to the development of persisting, often malignant tumors; (*b*) substances like croton oil and Tween 60, which have in common the effect of rapidly giving rise to a large crop of benign, often regressing papillomas; they are usually cumulatively referred to as "promoting agents," though there are differences in their administration requirements, as well as in their effects when tested without previous initiation. It is additionally well known that various other factors, particularly nutritional and endocrine changes, can severely modify the course of carcinogenesis: Clearly these actions must be distinguished from those described above. If we call "promotion" the second stage of carcinogenesis due to a full carcinogen as in (*a*), then we should not use the same term for the "croton oil effect" (*b*), because they are two different things. But since the term "promoting agent" is so much identified with the group of treatments that show the "croton oil effect," it would be more practical to call "promotion" the effect (*b*) and give a different name to the second stage of the action of full carcinogens (*a*) and speak of "developmental stage," "developing action," etc. If the term "promotion" should continue to be used indiscriminately, one should at least define in which sense it is used.

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EPITHELIAL TISSUE OF THE SKIN DURING CARCINOGENESIS[1, 2]

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SUMMARY

Effects of radiation and chemical carcinogens on the dorsal skin and vulva of mice and rats were compared. The chemical carcinogens induced cancers in descendants of treated cells, while radiation was followed by malignant changes in immigrating cells engaged in repair and exposed to the progressive vascular damage induced by irradiation. The more direct action of the chemicals was reflected in the histogenesis, yield, and duration of the induction time of tumors. Radiation did not induce a hyperplasia and induced fewer tumors after a considerably longer period than the chemicals. The relation of epithelial to connective tissue and vessels varied during the different phases of carcinogenesis, with the species, and with site in the same species. Dermal changes were related to papillomas during chemical carcinogenesis, but no consistent structural change occurred in the dermis during the development of morphological and functional anaplasia of epithelial components. In rats sarcomatous changes in the stroma of induced carcinomas occurred much more frequently than in mice. Carcinomas induced the same type of stroma at primary and secondary sites. Basal-cell carcinomas arose in the hair follicles of rats in unchanged dermis. The epidermal changes in radiation carcinogenesis appeared to be secondary to dermal alterations and these in turn were attributed to the slowly developing and extending vascular lesions.—NCI Monogr. No. 10: 509–529, 1963.

FOR THE experimental analysis of the interrelationship of the epidermis, dermis, and vascular constituents of the skin during formation of tumors, carcinogenic stimuli are required which act specifically on only one of the constituents. Thus the influence of the single changed component on the other unchanged components could be assessed with accuracy and certainty. In theory, such carcinogenic stimuli are available as short-ranged ionizing radiations, in the form of α-particles, electron beams, or β-rays from radioactive isotopes which can be used externally or intradermally. In practice, it has so far proved impossible to restrict the range of these radiations to either the epidermis or the dermis only.

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Hair follicles in the dermis present one of the great difficulties and the close contact between the basal epidermal layer and the superficial dermis with its blood vessels another. Since the ideal experimental conditions cannot yet be achieved, we have to rely on experiments in which there is a differential effect of carcinogenic stimuli on the various components of the skin. This differential effect may be due to the nature of the stimulus, *i.e.*, whether physical or chemical, to the different reactivity of homologous components in different species, or at different sites of the same species. We shall compare the histogenesis of skin cancers, in rats and mice, induced by chemical carcinogens and by ionizing radiations in the dorsal skin and relate the findings to observations on dorsal skin of mice grown in tissue culture and the vulval skin of rats and mice treated with chemical carcinogens.

The chemical carcinogens, benzpyrene (BP) and 9:10-dimethyl-1:2-benzanthracene[3] (DMBA), were applied, with a cotton wool swab, as 1 percent solutions in acetone once weekly to the dorsal or vulval skin of mice and rats. The experiments on the vulva were done jointly with Dr. C. P. Cherry. The irradiation experiments, in collaboration with Dr. J. W. Boag (*1*), made use of electron beams accelerated to 0.7 or 1.0 Mev in a van de Graaff generator. This procedure has the advantage of giving a fairly sharp and controlled fall off of the radiation dose at a given depth of tissue. In mice a circular area, 1 cm in diameter and in rats 2.5 cm, was exposed to a single dose of 8000 (mice) or 12,000 (rats) rads in 20 seconds or in rats to 2 doses of 2300 rads at an interval of 2 months. In further experiments, rats given a dose of 2300 rads were treated 15 months later with weekly applications of DMBA. A further group of rats was given a total dose of 6800 rads of 200 kv X rays over a field of 4 × 4 cm in 8 equal doses in 6 months. The essential carcinogenic effects of these irradiations were the same, and thus no detailed attention will be given to the role of dose and time factors.

With respect to the participation of the various tissues, three hypotheses of skin carcinogenesis can be distinguished: 1) A direct action on the epidermis is assumed for chemical carcinogens which induce an immediate malignant change (initiation) of single treated cells, transforming them into dormant tumor cells. Subsequent promoting action on such dormant cells enables them to form a colony of critical size, mainly by a delay in maturation of the stem cells (*2*). This model has found wide support in the investigation of skin tumors in mice. 2) The second hypothesis has been formulated mainly for the induction of cancers by irradiation (*3*). It relates the resulting cancers to the cells and tissues involved in the repair of the original radiation injury and particularly to the induced progressive vascular changes. The actually treated cells of the epidermis and dermis are lost, and the vascular injury conditions the immigrating neighboring cells indirectly to form cancers. 3) In chemical carcinogenesis in the mouse skin, the induction of an initial irreversible change in a field of the epidermis is accompanied by irreversible changes in the dermis. The latter persist and "promote" the malignant conversion of the epidermal changes (*4*).

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[3] *Chemical Abstracts'* nomenclature: 7,12-dimethylbenz[*a*]anthracene.

There may be more than only one mechanism leading ultimately to malignancy and indeed the progress to malignancy may be discontinuous and full neoplasia may result only from a number of independently occurring changes in the characteristics of the affected cells or tissues (*5*). Thus changes in growth rate, morphology, invasiveness, and loss of hormonal dependence may occur independently of each other during carcinogenesis. Evidence for such independent changes has been reported (*6*) for the induction of skin tumors in mice by chemical carcinogens. It is now established that the stages of hyperplasia and papilloma formation are neither necessary steps preceding carcinoma formation nor necessarily progress to full malignancy. In this study (*6*) only 11 percent of the papillomas ultimately became carcinomas.

Tissue interactions may vary with the particular step in the progression to full neoplasia, and it is important to distinguish the reversible steps such as hyperplasia and papilloma from the irreversible malignant conversion which itself encompasses a number of independent processes leading to morphological and functional anaplasia. The relation of dermal to epidermal and vascular changes during these various steps will be compared for two sites each in rats and mice for different methods of carcinogenesis.

In spite of the fruitful research based on the investigation of skin tumor formation in the mouse, it is unfortunate that other species have received but little attention. The mouse skin responds easily and quickly to various carcinogenic chemicals and also to some promoting agents, with the formation of warts. The mouse rarely produces sarcomas after external application of chemicals and only few basal-cell carcinomas. The rat is less sensitive to a great variety of chemicals, but produces basal-cell carcinomas (*7*) and sarcomas; it does not respond to promoting agents. Though rat skin requires a longer induction period than the mouse, it produces a greater proportion of carcinomas to papillomas than the mouse. Table 1 shows the incidence for the dorsal skin and illustrates the difference in response between the dorsal and vulval skin: In rats the incidence of carcinomas and sarcomas is significantly lower at the vulva than in the dorsal region. The figures for the vulva in rats and mice are similar, but the dorsal skin of the mouse resembles

TABLE 1.—Incidence of papillomas, carcinomas, and sarcomas induced by chemical carcinogens in the dorsal and vulval skin of mice and rats

Site	Number of animals	Percentage incidence of:		
		Papillomas	Carcinomas	Sarcomas
Dorsum	68 Rats	15	84	37
	48 Mice	38	48	0
Vulva	373 Rats	22	62	2
	98 Mice	23	57	0

the reaction of the vulva rather than that of the dorsal skin of rats. Rats and mice with carcinomas usually have papillomas which are not listed in the table. We prefer this assessment to a pseudoquantitative counting of macroscopic warts. Microscopic evidence shows that adjacent papillomatous regions often become confluent or are hidden by overlying warts. The number of macroscopic papillomas is thus no accurate measure of the original number of foci undergoing papillomatous changes.

The skin of mice and rats differs in regard to the hair coat which is finer and denser in the mouse than in the rat, and as regards the thickness of the dermis which is much greater in the rat than in the mouse. In relation to the length and width of the hairs, there is also a difference in the duration of the hair cycle between the two species. There is, however, very little difference in the structure of the dermal and vulval skin in rats as regards hair follicles, thickness of the dermis, and duration of the hair cycle. There is possibly more fat at the vulva than in the dorsal region, and the panniculus carnosus varies in thickness. Whether these differences have a bearing on the differences in tumor incidence shown in table 1 must remain mere conjecture at this stage of our knowledge.

Chemical carcinogens induce in the mouse and rat skin a hyperplasia characterized by a reduced rate of maturation and increased rate of proliferation (*8*, *9*). In mice, ulcerations occur particularly after repeated applications and when these are made in the resting phase of the hair cycle (*10*). Associated with the hyperplasia is a dermal change in which the dense collagen bundles are replaced by a thin-fibered cellular tissue and the elastic fibers are disarranged, clumped, or have disappeared. A slight, or in cases of ulceration, marked inflammatory reaction is often induced. In the mouse the closely spaced hair follicles are involved in the hyperplasia and the subsequent formation of papillomas. This leads to keratinization of the follicles instead of hair formation and the merging of changed hair pegs with radices formed by the epidermis and their joint formation of papillomas. In the rat the hair follicles are more widely spaced, and, though some undergo similar changes to those in mice, in many the hair sheaths start to proliferate and to form basal-cell tumors (figs. 1, 2, 3, and 4). In the rat, these changes in the follicles proceed while they are embedded in the normal dense connective tissue (fig. 1), and with the expansion of the tumor the dense dermis is pushed away. The replacement of dense dermal tissue by the new thin-fibered dermis progresses only slowly in the rat (figs. 6 and 8). The dense dermis may prevent radication of the epidermis (fig. 7) while the thin-fibered tissue allows the spreading of the epidermal radices (fig. 8) and also forms the stroma for the papillomas, whether these are pushing inward or outward. At this stage of carcinogenesis there are thus often correlated changes in the dermis and epidermis: radication and thin-fibered stroma, restriction of radication and dense fibrous tissue, as well an an expansion of proliferating hair follicles in unchanged dermis. There is no evidence that the epidermal and dermal changes are primary or secondary to one another, though mechanically it is plausible that a thin stroma allows expansion and a dense stroma acts as a barrier to expansion. The epidermal as well as the dermal changes extend in a varying degree over the whole treated region.

The next steps in carcinogenesis leading to morphological and functional anaplasia are not correlated with characteristic dermal changes. In hyperplastic and papillomatous regions, some of the morphologically anaplastic changes appear as abnormal divisions linked with formation of giant or multinucleate cells, abnormal, and incomplete maturation of keratinizing cells and their appearance outside their normal layers, *i.e.*, of immature cells in the superficial and of parakeratotic cells in the basal layers (fig. 5). These changes, like the loss of adhesiveness and change in shape of the polyhedral to a spindle type, the loss of polarity of cells and their ability to move in a basal as well as in a cuticular direction, their subsequent invasiveness and ability to spread through the basement membrane into the dermis (fig. 5) and ultimately into and through the panniculus carnosus, occur without any localized change in the dermal stroma; this remains the same in papillomatous regions and in morphologically anaplastic areas which usually extend over a great number of cells and even a great number of fields in the treated skin (fig. 9).

If late embryonic mouse skin is explanted to tissue-culture media containing DMBA or methylcholanthrene (MCA), hyperplasia of the epidermis is observed, and as early as 6 days after explantation (*11*, *12*) epidermal radices penetrate the panniculus carnosus. This happens when the dermis is reduced, as in treatment with MCA in air as well as when the dermis is preserved in treatment with DMBA in oxygen (*12*).

Functional anaplasia in the form of transplantability of the tumor to other organs (fig. 10) and loss of response to systemic factors appear at various stages in the development of morphologically anaplastic lesions without an obvious correlation to stromal changes. Yet once tumors are established, the type of stroma tends to be related to the tumor, particularly to its anaplasia and in particular to its growth rate and lack of differentiation as well as to the age of the tumor foci. Well-differentiated tumors (fig. 2) tend to have a denser fibrillar stroma, while more anaplastic types have little and mainly cellular stroma (figs. 3 and 4). Old foci are often surrounded by dense hyalinized stroma and here insufficient vascularization may account for the changes in tumor parenchyma as well as the stroma. Adjacent tumors of different types may have different proportions of fibrils to cells in their stroma (fig. 3). A tumor spreading to secondary sites such as the lymph nodes may in time induce the same type of stroma as at the primary site.

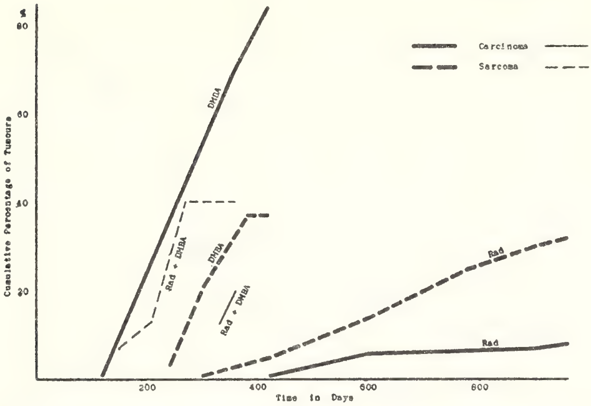
In the mouse the dermal change induced by treatment with chemical carcinogens tends to remain stationary at the formation of the cellular, thin-fibered stroma. In the rat—at least in the dorsal region—the change may progress to the formation of a fibromatous and a sarcomatous lesion, and this progress may coincide with that of the epidermal lesions to the formation of carcinosarcomas or at least of carcinomas with sarcomatous stroma (figs. 11 and 12). Sarcomas may also occur in regions with only minimal epidermal lesions and indeed induce there a concomitant hyperplasia, much as spreading carcinomas evoke in the overlying epidermis. There is thus evidence of a correlation of parenchymal and stromal changes in the late stages of neoplasia, evidence for the induction of a specific type of stroma by the tumor parenchyma, and evidence for the evocation of a hyperplasia in the epidermis by underlying sarcomas.

In their final stages tumors induced by radiation are indistinguishable from those induced by chemical carcinogens. They differ, however, in their histogenesis, in the duration of the induction period, in the incidence of the various types of tumors, and in the total yield of tumors. The main differences in the histogenesis of radiation-induced cancers and those induced by external application of chemicals are the killing and removal of the directly exposed tissue, followed by a progressive endarteritis and the formation of unstable scars. These are formed by the immigration of untreated dermal and epidermal cells into the lesion, and exposed to the unfavorable environment of progressive endarteritic changes the immigrating cells and tissues are undergoing the malignant conversion. In mice about half the cancers following irradiation are sarcomas (*13*, *14*), while in rats the sarcomas form 80 percent of all cancers (table 2). The vascular changes give rise to ulcerations (figs. 13, 14, and 15), and at the edge of the ulcers papillomas (figs. 13, 14, and 15) and carcinomas (fig. 16) are formed. In the rat the dermal scar varies between cellular and fibromatous stages and the scarring extends as the endarteritis spreads from the surface to the deeper blood vessels which were not directly exposed to irradiation. In the mouse the dermal changes in the scar precede the epidermal changes and may thus at least mediate the injurious consequences of vascular damage.

TABLE 2.—Induction of papillomas, carcinomas, and sarcomas in the dorsal skin of rats by DMBA, localized irradiation, and irradiation followed after 15 months by DMBA

Treatment	Number of rats	Percentage incidence of:		
		Papillomas	Carcinomas	Sarcomas
DMBA	68	15	84	37
Radiation	95	3	8	32
DMBA + radiation	15	0	20	40

The difference in the duration of the induction period for chemical and physical carcinogenic agents in the rat is illustrated in text-figure 1 which shows the cumulative percentage of tumor-bearing rats. The incidence of sarcomas is about the same for the two agents, but the induction period for 25 percent sarcomas is 200 days for DMBA and 800 days for radiation. A similar prolongation of the induction period is seen for carcinomas, which occur in a much smaller proportion of rats after radiation than after DMBA painting (table 2). Previous exposure to



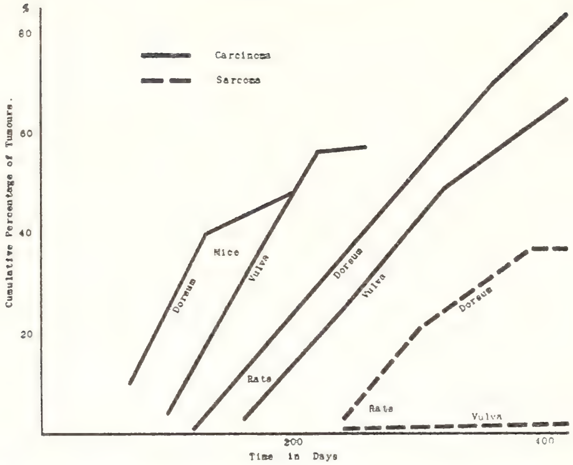
TEXT-FIGURE 1.—Tumor induction in the dorsal skin of rats by DMBA, radiation, and by both.

a dose of radiation causing permanent epilation, but no tumor formation, slightly shortens the induction period for sarcomas as compared with DMBA painting of intact skin, yet greatly prolongs the induction period for carcinomas and reduces their incidence. In mice the tumor yield after external β-irradiation is 12 percent in 125 animals and the induction period for 10 percent carcinomas is 450 days instead of 75 days for chemical carcinogens. Combination of chemical carcinogens with irradiation increases the incidence and shortens the induction period of sarcomas (text-fig. 1).

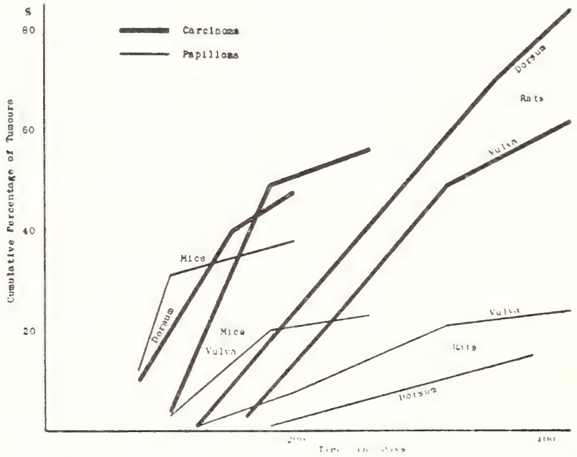
The histogenetic as well as the statistical evidence supports the idea that cancers are formed "directly" by the descendants of cells and tissues treated by chemical carcinogens, while radiation induces cancer indirectly by its effects on the blood vessels, which in turn cause the malignant conversion of untreated cells engaged on repair of the radiation damage. The indirect effect is slow, occurs more rarely, and actually depends on a balance of injury and repair: It does not occur with small doses causing too little injury and not with big doses which prevent repair.

This does not mean that the direct carcinogenic action of chemicals is a continuous process independent of outside influences on the treated cells. This can be illustrated by differences in tumor yields, in tumor type, duration of induction period for different species, for different sites in the same species, and for the influence of additional systemic treatment by altering the endocrine state of the animals.

DMBA painting induces sarcomas in the dorsal region of a third of the rats, but in the vulva in only 2 percent (text-fig. 2, and table 1), while sarcomas are not induced by the same treatment in either the dorsal region or the vulva of mice. The yield of carcinomas is greater for both sites in the rats than in the mice, but the induction period is longer. For the same type of treatment the proportion of induced papillomas to carcinomas varies with the species and to some extent also with the site in the same species (text-fig. 3). In mice papillomas appear in the dorsal



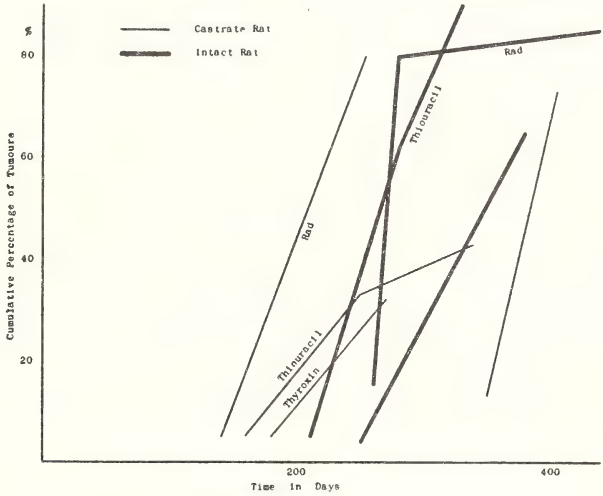
TEXT-FIGURE 2.—Tumor induction by chemical carcinogens in the dorsal skin and vulva of rats and mice.



TEXT-FIGURE 3.—The proportion of carcinomas and papillomas induced by chemical carcinogens in the vulva and dorsal skin of rats and mice.

skin at the same time as carcinomas do in other animals, while in rats the carcinomas precede the papillomas in the dorsal region. In the vulva of mice papillomas and carcinomas are observed at the same time, but in rats papillomas appear earlier than carcinomas in the vulva. The proportion of papillomas to carcinomas is highest in the dorsal region of mice, lowest in the same site for rats, and intermediate and about equal for the vulva of rats and mice. This indicates that the tumor progression varies not only with the species but also to some extent with the site, though the histology of dermis and epidermis at these sites may appear very similar.

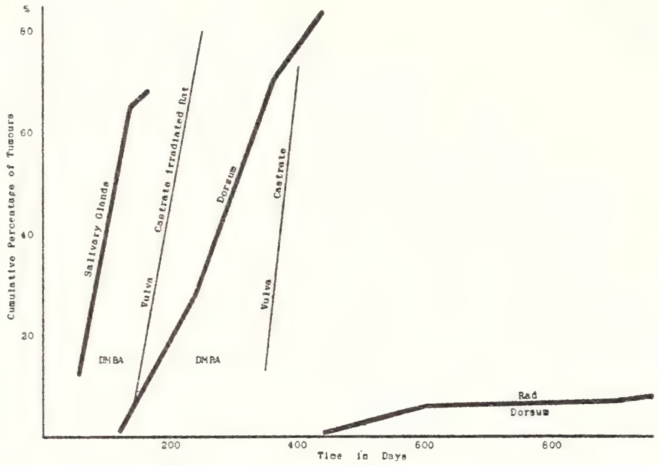
For the same site additional systemic treatments influence the induction period and the yield of tumors. Text-figure 4 shows the incidence of carcinomas of the vulva in rats after such experimental alterations; castration increases the induction period as compared with intact rats. In intact rats radiation of the pelvic region or the administration of thiouracil in the drinking water does not appreciably affect the length of the induction period and the tumor yield. Pelvic radiation of castrated rats markedly decreases the duration of the induction period and slightly increases the tumor yield. The administration of either thyroxin or thiouracil to castrated rats decreases the duration of the induction period and the tumor yield. The induction of vaginal sarcomas by DMBA painting is even more strikingly affected by hormonal treatments, and in the same rat the treatments may have different effects on the induction of vulval carcinoma and vaginal sarcomas. The effect on the duration of the induction period by thiouracil equals that by thyroxin, though the first might be expected to slow down the turnover of cells and the second to speed it up.



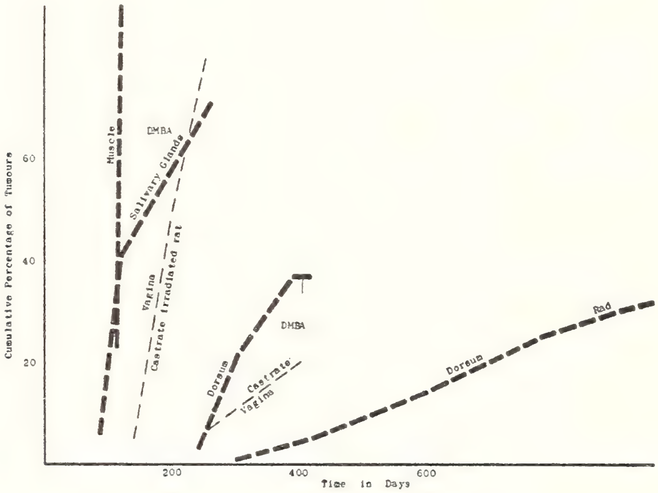
TEXT-FIGURE 4.—Induction of carcinomas in the vulva of intact and castrated rats by DMBA and additional radiation or administration of thyroxin or thiouracil.

There are also striking differences, depending on the type of carcinogenic stimulus and the form of application, in the length of the induction period. Injection of DMBA causes an early appearance of carcinomas in salivary glands (text-fig. 5) presumably because of a depot of the chemical at the site of injection that exerts a continuous stimulation. Weekly paintings of DMBA induce carcinomas in the dorsal region and the vulva, varying in duration of the induction period with the site and the state of the animal, while radiation given in a single dose or in repeated doses over 6 months produces few carcinomas and very slowly.

A similar variation in the induction time for sarcomas is illustrated in text-figure 6. The injection of DMBA into the salivary glands and the thigh muscles causes the shortest induction period. There is a striking



TEXT-FIGURE 5.—Induction of carcinomas in the salivary glands, vulva, and dorsal skin of rats by DMBA and radiation.



TEXT-FIGURE 6.—Induction of sarcomas in muscles, salivary glands, vagina, and dorsal skin of rats by DMBA and radiation.

difference in the duration of the induction of vaginal sarcomas in castrated rats without further treatment and those given additional pelvic radiation. Again radiation alone does not produce sarcomas as quickly.

Even shorter induction periods have been recorded: In tissue cultures of mouse skin exposed to chemical carcinogens the panniculus carnosus was invaded after 6 days (*11, 12*); treated fetal mouse skin transplanted to untreated sites induced carcinomas in 34 days (*15*); and the first growth of a subsequently verified carcinoma was observed 2.5 weeks after the transplantation of untreated adult epidermis to a pretreated subcutaneous site (*16*). Interference with the vascular supply is the common factor in these transplantation and explantation experiments and might be a factor in the shortening of the induction period, rather than the removal of these tissues from the forces which normally regulate their growth and may be expected to control their response to carcinogenic stimulation.

This short survey shows that carcinogenic stimuli may act directly on tissues which subsequently become malignant or indirectly create conditions in which unexposed cells and tissues undergo a malignant change. We have tried to correlate structural changes in the dermis and epidermis and its appendages, but encountered a variety of relationships depending on species, site, and the particular step in the neoplastic process considered. These conflicting observations may be due to the fact that, in spite of the same morphology, tissues may exert a different chemical or inductive influence. That endocrine changes and other systemic factors materially affect carcinogenesis suggests that more subtle forces may be at work which are not obvious morphologically. That the parenchyma and mesenchyma are necessary for each other and have a specific effect on each other is well known from experimental embryological observations (*17*). Some of these inductive qualities on one another are carried over into adult life and may thus influence in a positive and negative manner the induction of cancers.

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PLATES

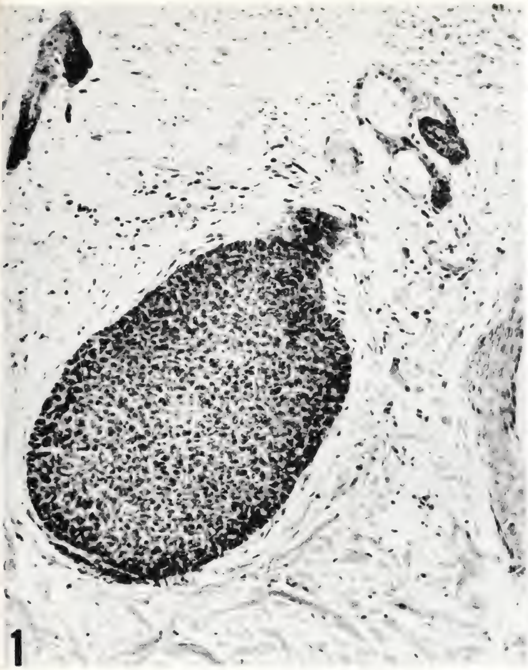
PLATE 55

FIGURE 1.—Dorsal skin of rat 7 months after weekly paintings with DMBA. Basal-cell tumor replaces a hair follicle in deeper part of dermis, which is formed by unchanged dense connective tissue. × 140

FIGURE 2.—Rat skin 14 months after exposure to 8 doses of 850 rads each in 6 months. Large basal-cell carcinoma has arisen from hair sheaths and hairs are replaced by concentric layers of parakeratotic cells (h). Tumor strands are separated by well-formed and fairly dense fibrillar stroma. × 240

FIGURE 3.—Vulval skin of rat 11 months after weekly paintings with DMBA. Squamous-cell papilloma with fibrillar stroma is found next to a slightly anaplastic basal-cell carcinoma with little and predominantly cellular stroma. × 210

FIGURE 4.—Part of figure 3 at higher magnification, showing sparse stroma and numerous cell divisions in tumor. × 475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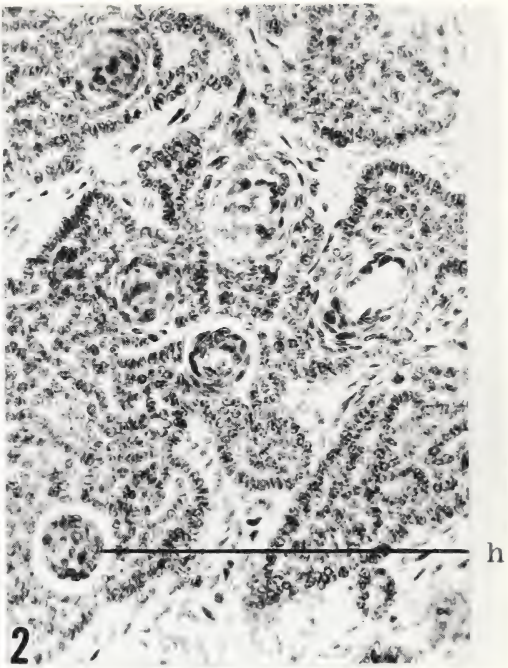
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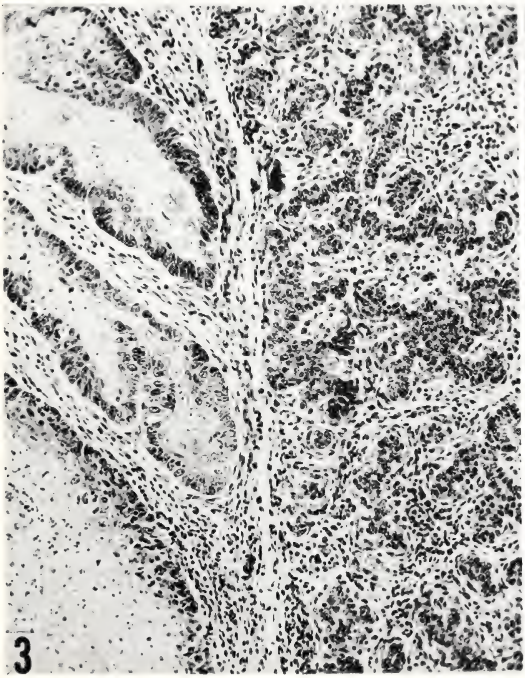
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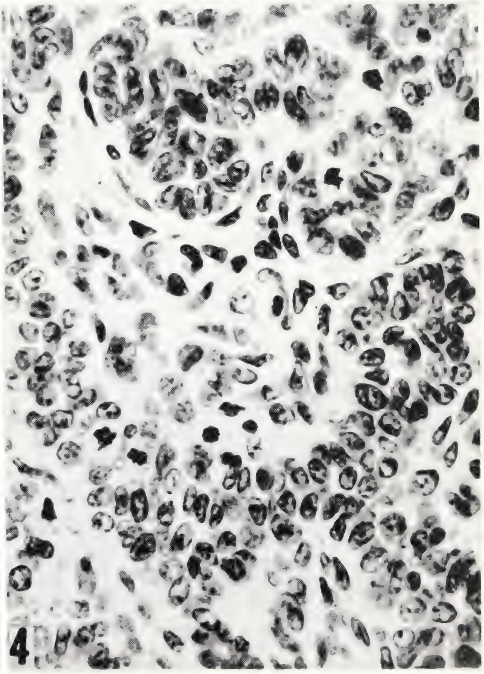
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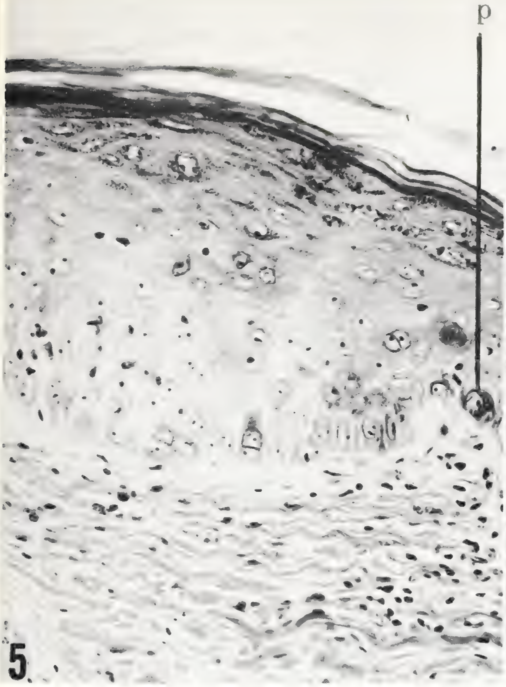
Plate 56

Figure 5.—Dorsal skin of rat 7 months after weekly paintings with DMBA. Epidermis is hyperplastic, shows bi- and multinucleate as well as parakeratotic cells (p) in basal layer. Underlying dermis is cellular and thin fibered. × 280

Figure 6.—Dorsal skin of rat 8 months after weekly applications of DMBA. Extensive epidermal radication is associated with replacement of the usually dense superficial dermis by a cellular and thin-fibered tissue. Deeper layers of dermis are unaltered, hair follicles and sebaceous glands persist. × 30

Figure 7.—Dorsal skin of rat 6 months after weekly paintings with DMBA. Epidermis is hyperplastic, but shows only slight and short radication. Except for a thin superficial zone dermis has retained its normal dense structure. Hair follicle on *right* is in telagen and consists of a small papilla, some sebaceous cells of fairly normal type, and a keratinized superficial zone. × 115

Figure 8.—Part of figure 6 at higher magnification, showing cellular stroma and regular size and normal stratification of epidermal cells even in radices. × 120



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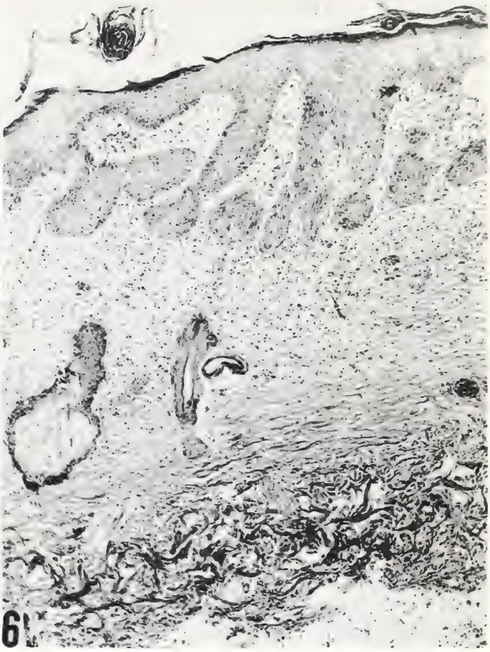

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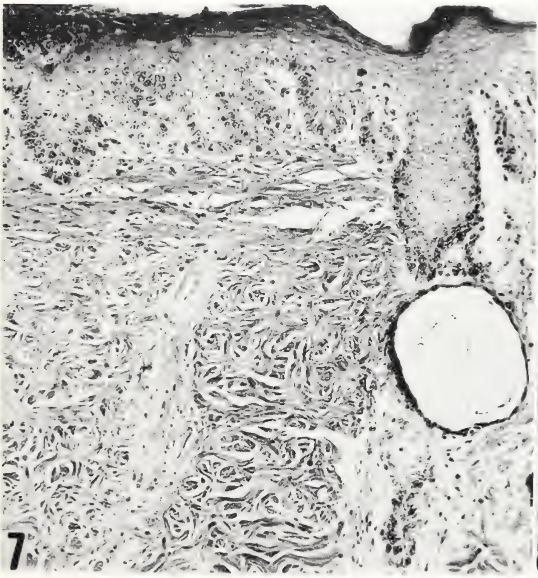

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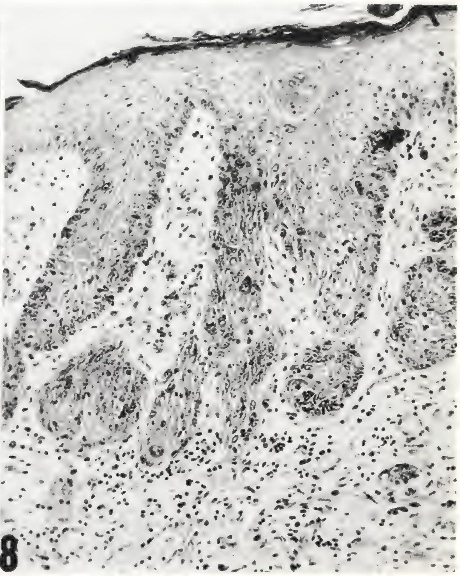

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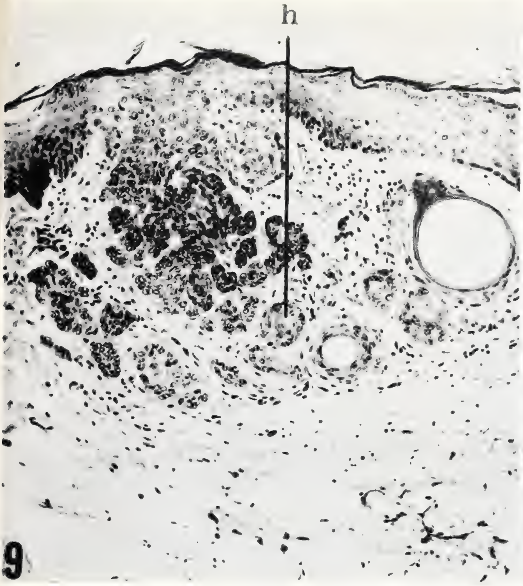
PLATE 57

FIGURE 9.—Vulval skin of rat 9 months after weekly paintings with DMBA. Epidermis is hyperplastic and cells of hair follicles show proliferation, invasiveness, loss of stratification, and adhesiveness. In some formations (h) central cells keratinize and these as well as neighboring formation resemble structures seen in basal-cell carcinoma of figure 2. × 120

FIGURE 10.—Dorsal skin of rat 13 months after weekly paintings with DMBA. A fairly anaplastic carcinoma is composed of spindle-shaped, actively dividing cells with little adhesion and no stratification, and has invaded perineural spaces and spreads from there. × 210

FIGURE 11.—Dorsal skin of rat 9 months after weekly paintings with DMBA. Fairly dense stroma around squamous-cell carcinoma has changed to fibrosarcoma in some regions. × 140

FIGURE 12.—Dorsal skin of rat 30 months after single exposure to 12,000 rads. Cellular stroma surrounding a carcinoma has undergone malignant change and shows numerous cell divisions. × 220



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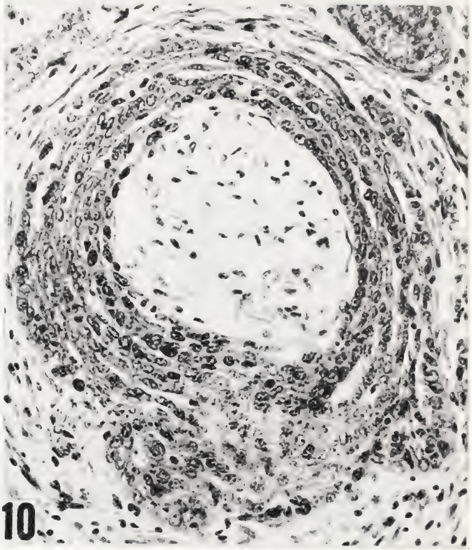

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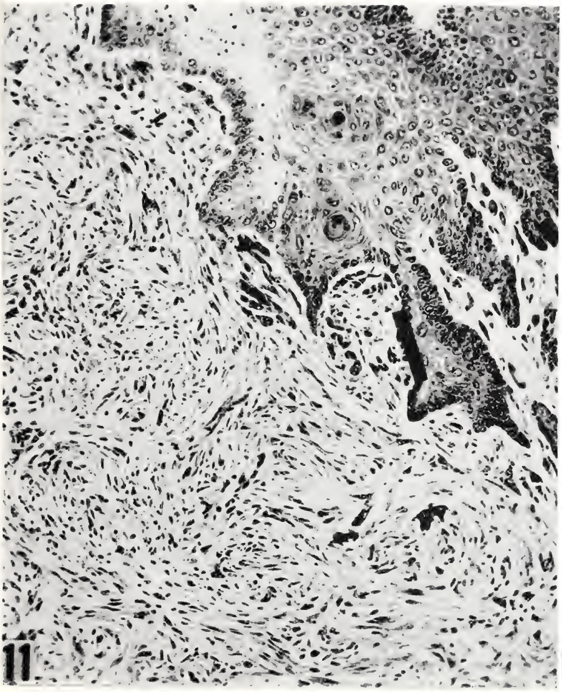

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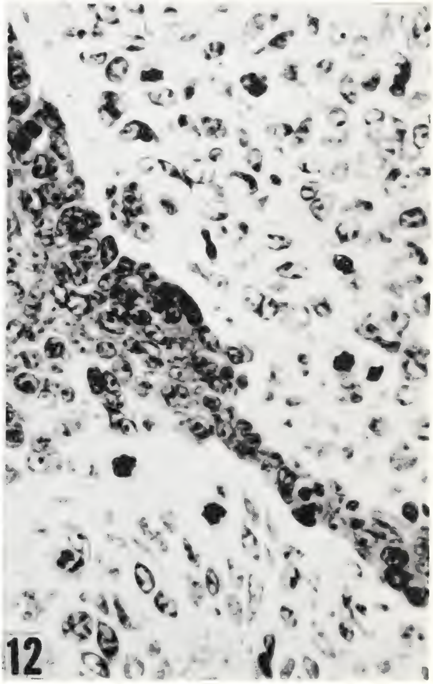

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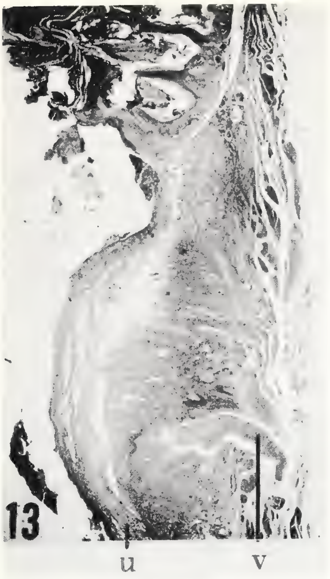
Plate 58

Figure 13.—Dorsal skin of rat 20 months after single exposure to 12,000 rads. At edge of radiation ulcer (u) a papilloma is seen. Grossly dilated vessel (v) with stagnant blood is seen in center of ulcer. × 15

Figure 14.—Papillomatous region of figure 13 at higher magnification. Dilated vessels (v) are seen at base of papilloma. × 30

Figure 15.—Dorsal skin of rat 32 months after exposure to 12,000 rads, showing a radiation ulcer (u), with dilated blood-filled vessels (v), and at edge of ulcer a papilloma. × 15

Figure 16.—Dorsal skin of rat 16 months after 2 exposures to 2300 rads each at an interval of 2 months. At edge of ulcer (u) squamous-cell carcinoma has formed which has just invaded panniculus carnosus (pc). × 15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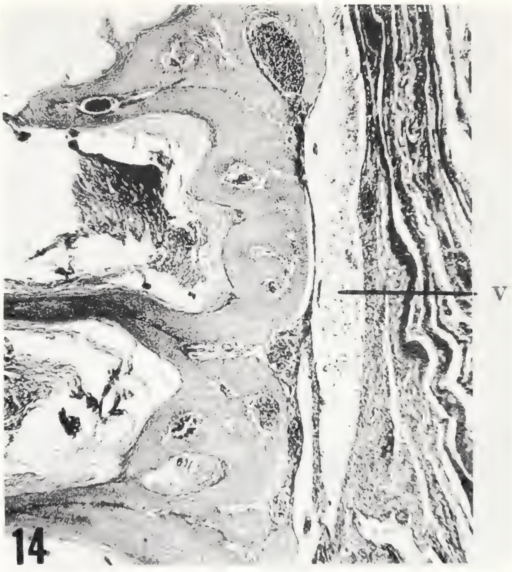
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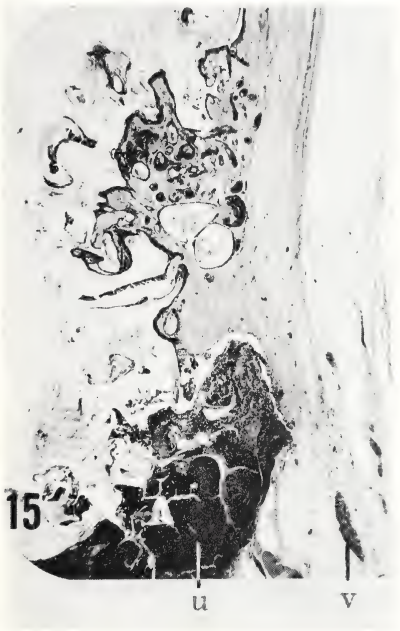
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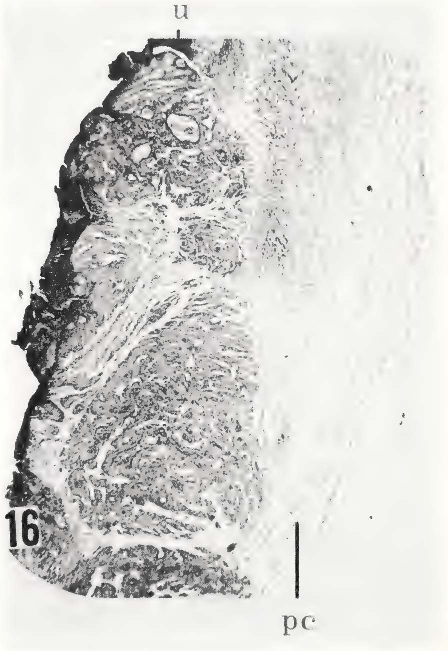
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THE ROLE OF THE STROMA IN EPIDERMAL CARCINOGENESIS[1, 2]

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SUMMARY

The mechanism of chemical epidermal carcinogenesis cannot be satisfactorily explained in terms of a direct carcinogen-epithelial-cell reaction. During the latent period of carcinogenesis permutation of the stroma (dermis and subcutis) occurs and can be demonstrated by histological and experimental methods. When epidermis is transplanted from skin that has received a full carcinogenic treatment, it does not develop tumors in its new site. At the carcinogen-treated site tumors may originate from epithelium that has not been in contact with carcinogen; that the hair follicles are concerned in this is unlikely. Carcinoma can be induced in subcutaneous grafts of skin with much smaller doses of carcinogen that are necessary for carcinogenesis *in situ*.—NCI Monogr. No. 10: 531–537, 1963.

CANCER, in general, is a disease process in which the cells composing the tumor can be related by histopathologists, with a great deal of confidence, to various normal cell types in the body. Nobody now doubts, for instance, that the cells of which gastric carcinoma is composed are the progeny of the stomach epithelium. It seems remarkable that it is still less than a century ago since this apparently self-evident fact was generally accepted. Even Virchow, the founder of cellular pathology as we have since understood it, was reluctant to accept the idea that the cells of malignant tumors of epithelial tissues were epithelial cells and not a special type of metaplasia of connective-tissue cells. This particular controversy is now finished, however, and I think we can assume that no informed observer now seriously doubts that skin cancers are composed of cells derived from the epithelial (epidermal) cells. They not only look like epidermal cells, but they produce keratin and have many of the other biological and chemical properties of epidermal cells.

It is therefore natural that, when methods of experimental induction of skin cancer became available, the explanation of its mechanism should

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

[2] Much of this work was supported by the Birmingham Branch of the British Empire Cancer Campaign, in whose laboratories some of the work was done, and by the Medical Research Fund of the United Birmingham Hospitals.

have been sought for primarily in a direct effect of the carcinogen on the intrinsic properties of the epithelial cell. The great difficulty to be overcome in the application of this explanation is the long "latent" period before tumors develop. We used to think that long-continued treatment with carcinogens was necessary, but we now know that one application of a carcinogen is sufficient if it is followed up by treatment with a cocarcinogen (*1*). On the basis of a direct carcinogen-epithelial-cell reaction, it therefore became necessary to postulate a "latent" or "dormant" cancer cell (*2*). But on this postulate one finds it difficult to understand why the dormant cell of dibenzanthracene (DBA) "sleeps" twice as long as that of dimethylbenzanthracene (DMBA). Another great difficulty is that during the latent period there is continuous attrition and exfoliation of epidermis, with regenerative replacement involving multiplication of cells that nevertheless reveal no neoplastic properties until the latent period is completed. These considerations apply to both the superficial epidermis and its epithelial appendages, the hair follicles, and sebaceous glands.

The dermal stroma, on the other hand, is probably more permanent. When systematic histological studies were made at intervals throughout the latent period, it was found that changes in the dermis and subcutis were continuously progressive (*3*). Furthermore, the rate at which such changes developed was related to the potency of the carcinogen; stromal changes advanced more rapidly with, for instance, methylcholanthrene (MC) than with DBA. This contrasts sharply with what happened to the epidermis in which, after an initial hyperplasia, there was little additional difference, presumably because of an equilibrium between attrition and regeneration, until the tumors themselves began to appear.

The stromal changes can be detected clinically by the thickening of the skin and macroscopically by the thickening and also congestion, with increased prominence of vessels on the under surface of the reflected skin. Histologically, the most striking change is the replacement of the normal, coarse, interwoven collagen fiber structure by fine nonrefracticle fibers that tend to be orientated parallel to each other and the skin surface. This change did not occur, in my experience, with noncarcinogenic irritants, though there was a suggestion of it with the cocarcinogen croton oil. A similar observation was briefly referred to by Wolbach (*4*) and confirmed by Howes (*5*), Ma (*6*), Gillman *et al.* (*7*), and Maltoni and Prodi (*8*).

A gradual change in the elastic tissue to a granular fragmented form is also seen, and it was thought by Orr (*3*) that the tumors originated at sites in the skin where there were gaps in the dermal elastin. Gillman *et al.* (*7*) regarded the abnormal material not as true elastin, but as an elastotic degeneration of collagen. Another striking change in the dermis of carcinogen-treated mice is an increase in the number of mast cells, but estimation of the significance of this increase is difficult, for it occurs also with various noncarcinogenic irritants. The studies of Maltoni and Prodi (*8*) represent the most comprehensive account up to now of the morphological and histochemical changes in the dermis of carcinogen-treated animals of various species. These authors have pointed out that there is an accumulation of mucopolysaccharide in the dermis of the carcinogen-treated rabbit, a phenomenon, however, that rarely occurs in other species, including the mouse. Biochemical study of the collagen of carcinogen-treated skin has not revealed changes that are likely to be of significant relevance (*9*).

The possibility that stromal changes are of pathogenetic importance in the origin of epithelial, and more particularly epidermal, cancer is by no means a new idea. It was implicit in the old chronic-irritation theory of Virchow and in the tissue-tension theory of Ribbert and others. It was stressed in the studies of irradiated human skin by Wolbach (*10*) and in those of various human "precancerous" lesions by Vernoni (*11*). The observations of Kreyberg (*12*) on the vascular changes in the tarred skin of mice, and those on which Handley (*13*) based his lymphstasis theory of carcinogenesis, also emphasized the stromal changes antecedent to the onset of epithelial neoplasia.

I have suggested the term "permutation" (*14*) for the total series of changes occurring in the stroma of a preneoplastic site. This term includes all the changes—morphological, functional, fibrillary, cellular, and vascular—that occur in the altered stroma as a whole. At one time I believed that the vascular deficiencies of the permutated stroma might be the essential element in the situation; there is support for this in the vessel-injection studies of Kreyberg (*12*), in the effects of vasoconstrictor drugs accompanying carcinogen treatment (*15, 16*), and in the focal changes in *p*H demonstrable by the intra-vitam injection of phenol red (*17*). Consideration of this sort would support the idea that tissue hypoxia is an important factor in carcinogenesis, an idea which has been entertained by many of us since the classical work of Warburg of 40 years ago.

It has appeared to some workers that the alteration in the texture of the dermis may reduce its capacity to resist invasion by epidermal cells, which is the earliest criterion by which carcinoma can be recognized. But invasive growth *per se* is by no means necessarily neoplastic, and, as Wolbach (*18*) has pointed out, the developing hair follicle penetrates the *normal* dermis very rapidly—more rapidly, indeed, than does a carcinoma. It therefore seems more rational to me to take the provisional view that the stromal permutation affects the epithelium as a result of interference with metabolic exchange, so that the epithelium remains viable but deprived of essential organizational controls. The difficulty of maintenance of cells in tissue culture without their undergoing neoplastic change (*19*) seems relevant, as do some of the phenomena associated with the cessation of the healing of wounds. It may even be that some types of isolated mammalian cells are more like cancer cells than "normal" cells.

We have in this department in recent years attempted to allocate the roles of epidermis and stroma in carcinogenesis by transplanting skin components during its course. Billingham, Orr, and Woodhouse (*20*) painted an area of skin over the *right* scapula of mice with MC for 12 weeks. After such treatment, it could be anticipated that most of the mice would later develop tumors. When pure epidermal grafts, or thin split-skin grafts, were taken from this area and transplanted to a half-thickness bed in the skin over the *left* scapula, they did not develop tumors. Even if such grafts were subsequently given cocarcinogenic treatment with croton oil, the number of papillomas was small, no greater than might be expected with croton oil alone (*21*). In all these experiments numerous tumors—papilloma and carcinoma—developed on the original MC-treated *right* scapular site, even when it was completely denuded of epidermis and left to resurface itself by ingrowth from the periphery or immediately covered by grafts of untreated tail skin.

An obvious complication of these experiments was that when the grafts were cut, the proximal parts of the hair follicles were left *in situ,* and it might have been from this source the tumors originated. However, I do not regard this as likely for two reasons. First, the tumors were all squamous; they never showed basal-cell, trichoepitheliomatous, or sebaceous structure, though induction of tumors of these types with MC in oily solution is possible. Secondly, a histological study of the early effects of DMBA showed that this hydrocarbon rapidly produces complete necrosis of the superficial epidermis, hair follicles, and sebaceous glands; hair follicles are subsequently differentiated *de novo* from the epidermis that migrates in from the periphery and under the slough (*22*). That hair follicles could be "neo-differentiated" in this way had been shown by Breedis (*23*), and we no longer have to regard hair follicles as embryologically differentiated, irreplaceable structures. I have no equally conclusive evidence in the use of MC, but Gillman *et al.* (*7*) believe that the hair follicles are also destroyed by the MC, and from my own observation and inferences I would agree with them. I am therefore disinclined to think that the residual hair follicles in the deep dermis are the source of the tumors at the donor site in the grafting experiments described. It is cogent in this context to remember that in experiments by Shubik and Della Porta (*24*), where large doses of hydrocarbon were used which caused extensive destruction of the epidermis and its appendages, tumors developed much more rapidly than in experiments where the conventional, less dramatically necrogenic dosage was used.

To ascertain whether it is necessary for the epithelium to come into contact with carcinogen, King and Orr (*25*) inserted paraffin wax pellets containing MC into the subcutaneous tissue of the flank of mice. After varying periods of time, the most successful being 2 weeks, these pellets were withdrawn, and thin grafts of untreated ear or tail skin were introduced into the sacs that the pellets had occupied. Under these circumstances both sarcomas and squamous carcinomas developed. The origin of the carcinomas had nothing to do with hair follicles, which did not survive in these grafts, though they persisted (and indeed appeared to grow and develop) in the cysts formed from control grafts into untreated subcutaneous sites. It was expected that there would be no detectable

MC in the tissue surrounding the pellet sacs, but in a series of experiments to check this point we found that small amounts of 30 to 60 μg might have been present, but they never persisted for more than 3 days after the removal of the pellet. King and Orr (*25*) were therefore unable to claim that the subcutaneously grafted epidermis had never been in contact with MC. Grafts inserted into pellet sites 4 days after the removal of the pellet gave negative results.

It therefore became necessary to test directly whether amounts of MC of this order would produce carcinoma under these experimental conditions. Dr. A. T. Spencer (unpublished results) has carried out a series of experiments using a technique that he has devised for applying accurately measured amounts of MC to tail-skin grafts before cutting them. The grafts were inserted subcutaneously in the flank. Under these circumstances both carcinoma and sarcoma occur with doses of MC as small as 20 μg. This was true whether the grafts were cut immediately after MC treatment or after a few days' interval; the highest yield of carcinoma appeared to occur if the tail was left intact for 1 day before the grafts were cut. The MC penetrated the hair follicles and sebaceous glands rapidly, so that in practice it was impossible to cut the grafts while the carcinogen was confined to the superficial epidermis. Dr. Spencer has confirmed that tail skin is refractory to carcinogenesis if left *in situ*. Since these experiments are still in progress, it would be premature to discuss their implications.

The complexity of the epidermal-stromal relations is further emphasized by the recent observations of Ashley (*26*), who found that whole skin grafted subcutaneously after 6 to 12 weeks of carcinogenic treatment did not develop tumors. This duration of treatment would certainly be fully adequate for carcinogenesis *in situ*, and Billingham, Orr, and Woodhouse (*20*) found that the transplantation of MC-treated whole skin to another *surface* site was followed by the appearance of tumors. A tentative explanation of Ashley's results might be that subcutaneous transplantation restores the vascular and nutritional conditions, which are impaired by permutation of the dermis, whereas in the surface grafts permutation persists in the grafted dermis. There is, however, another conclusion, which can be drawn on firmer grounds from Ashley's experiments; he would seem to have shown decisively that the epithelium of his grafts was not committed irreversibly to neoplasia, even in the sense of initiation. If this is true, it represents a further serious objection to the view that carcinogenesis can be explained on the basis of a direct reaction between the chemical carcinogen and the epidermal cells.

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ANATOMY AND PATHOPHYSIOLOGY OF SKIN TUMOR CAPILLARIES[1, 2]

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SUMMARY

The minute vessels of malignant experimental tumors and of human skin neoplasms are strikingly abnormal. There is a vigorous vascular proliferation at the border of rapidly growing tumors, and a marked increase, dilation, tortuosity, cross-anastomosing, and distortion of capillaries in solid cancers and under human premalignant skin lesions. However, the presence of a large number of capillaries in tumor tissue does not necessarily imply an abundant supply of nutrient materials. Evidence is presented that this rich vascular bed is mechanically and functionally inefficient, and results in very slow blood flow and chronic tissue hypoxia. The implications of these observations for the development and growth of tumors and the problem that sluggish tumor circulation poses for cancer chemotherapy are discussed.—NCI Monogr. No. 10: 539–559, 1963.

STUDIES ON the anatomy of tumor blood vessels date back over 100 years (*1*) particularly to the detailed observation of the father of modern pathology, Rudolph Virchow (*2*). As with much of cancer research, the earliest experimental investigations were carried out on skin, especially on the vascular changes induced by ionizing radiation and neoplasia subsequent to this physical carcinogen (*3–6*). The early investigators noted significant anatomical abnormalities in the arrangement of blood vessels in and around tumors and postulated a functional relationship between these alterations and the development of neoplasms (*3*, *6*, *7*).

ANATOMICAL OBSERVATIONS

Developmental Anatomy of Tumor Blood Vessels

The development of the transparent ear chamber technique (*8*), combined with advances in contrast media radiography, paved the way for

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

[2] Supported by grants C-4232 and C-6031 from the National Cancer Institute, National Institutes of Health, Public Health Service.

a series of excellent studies on the developmental anatomy of tumor blood vessels.

Early investigations (*9, 10*) with contrast media confirmed *in vivo* the anatomical observations of Goldman (*6*) on the structure of the blood vessels of tumors. The major advances of our present knowledge on the vascularization of malignant tumors were made by Ide *et al.* (*11*), Algire and Chalkley (*12, 13*), and Algire *et al.* (*14*). Because of their investigations, it is now possible to present a sequential picture of the development of the blood vessels of tumors. Their findings may be summarized as follows:

1) In the normal course of events, capillaries begin to proliferate 6 to 7 days after a normal homologous tissue is transplanted into a transparent chamber. Gradually a differentiation into arterioles, prearterioles, capillaries, venules, and veins takes place, until the vascularization of the tissue is normal (fig. 1).

2) After an immunologically compatible sarcoma or carcinoma has been implanted, proliferation of vessels begins earlier, on the 3d day, and is much greater than in control implants of normal tissues. The arteries in the host tissues become engorged, thickened, tortuous, and begin to give off large shoots. Concomitantly, the intratumoral (transplanted) arteries become wide and coiled. At the advancing border of the tumor, the veins show coronation, looping, and budding. The vessels sprout into the tumor mass and anastomose with the buds of vessels of the tumor explant. Most of the newly formed vessels consist of endothelial channels, of wide diameter, with little or no differentiation into arterioles or venules; these vessels anastomose freely in all directions and form an intricate network. Some of the new vessels are solid strands of endothelium that later canalize; many are collapsed but demonstrably patent. The sinusoidal capillaries forming the bulk of the vessels show great variation in blood flow and little contractility (fig. 2).

3) Proliferation of endothelial capillaries continues during the growth of the tumor. Growth, however, does not begin until several days after the explant has been vascularized. [This suggests that there is a production of a blood vessel growth-stimulating substance by the tumor (*11*).]

4) Most vessels collapse instantly when the animal dies or the arterial blood pressure is moderately reduced.

5) Most carcinomas and sarcomas examined have the abnormal, rich, vascular pattern described. It appears that the ability of transplanted cell masses to grow as sarcomas is directly related to their capacity to induce continuous endothelial proliferation. By contrast, normal fibroblasts and plasma clots produce only a slight vascular reaction (*14*).

6) As opposed to carcinomas and sarcomas, transplanted melanomas show considerably less disordered vascular patterns (*15*), while transplanted lymphomas evoke only a normal revascularization response (*16*) (fig. 3).

Direct examination *in vivo* of the sequences of vascularization of transplanted, malignant mouse tumors shows a vasculature identical to that described histologically in human material. There is, however, the striking exception of the malignant lymphoma that seems to act as a simple infiltrate. Recent studies by Waters and Green (*17*) with granulosa-cell tumor lead to the same conclusions as those just cited.

Blood Vessels of Human Tumors

Several investigators have studied the vasculature of human tumors. These studies have largely utilized either angiography or postmortem injections of organs with radiopaque dyes and subsequent radiography. The tumors of bone, liver, and lung have been examined primarily. Angiography has been useful mainly in bone tumors (*18–21*). In general, observations on human tumors are in agreement with those noted in the transparent-chamber transplants. There is a disordered proliferation of irregular vessels of variable caliber at the border of the tumor. The radiopaque dye appears very rapidly in a rich and irregular complement of dilated, cystic veins, which suggests the presence of many arteriovenous shunts (*21*). The feathery and puddling appearance of the dye in the body of the tumor shows a rich and anastomosing capillary network. The long persistence of the dye in the tumor capillaries suggests low overall rate of flow through the tumor mass (figs. 5 and 6).

Injections of liver tumors (*22*, *23*) show a puddling of dye, bizarre branching of arteries, many random anastomoses, and a blood supply derived mainly from the hepatic artery and separate from the portal system (fig. 4). In the lung (*24*), marked arterial proliferation at the edges and large vascular lacunae, lined with endothelium and derived from the bronchial artery, have been demonstrated.

ANATOMY OF HUMAN SKIN TUMOR CAPILLARIES

The capillaries supplying the skin and cutaneous appendages can be easily seen in frozen sections prepared by the Gomori technique for demonstrating alkaline phosphatase. This method, showing even collapsed or blocked vessels of capillary caliber, is superior to the conventional benzidine and silver impregnation techniques. Alkaline phosphatase activity appears to be intense only in the endarterial or capillary endothelium, while the walls of venules and larger arterioles show either little or no enzyme activity.

A variety of cutaneous human premalignant and malignant lesions was examined, and a number of benign neoplasms and inflammatory skin diseases were investigated for control purposes. The tissue was fixed for several hours in 10 percent cold, neutral-buffered formalin, frozen sections were cut between 30 and 50 μ in thickness, and a Sartorius model 22 freezing microtome was used. The sections were prepared by the Gomori method for alkaline phosphatase.

The normal anatomy of cutaneous capillaries has been described by Ellis, Kopf, and Winklemann (*25–27*). In sections stained for alkaline phosphatase, capillary loops entering the dermal papillae are seen, with a rich capillary plexus surrounding the cutaneous appendages (fig. 7).

In a variety of benign inflammatory and hyperplastic skin disorders, only minor changes in capillary structure were noted. In inflammatory diseases, the capillaries were generally sparse and pushed aside by edema and inflammatory infiltrate (fig. 9); in psoriasis the typical changes consisted of a regular arrangement of capillary loops, slightly dilated and tortuous at the top, giving a "Swiss cheese" appearance in horizontal section (fig. 11). In benign tumors such as nevi or molluscum contagiosum, the vessels sparsely surrounded the epithelial-cell strands or formed coronal sinuses, without any increase or abnormality of structure (figs. 8 and 10).

In sections of malignant skin neoplasms, the capillary structure is strikingly altered. There is a significant increase in the number of visible capillaries, which are elongated, dilated, and tortuous, showing many anastomoses and cross-linkages. The altered vessels surround and extend beneath the proliferating islands and strands of tumor cells of basal-cell carcinomas (fig. 12). In 15 such specimens, no capillaries were found within the neoplastic epithelium even when the tumor strand width exceeded 300 to 350 μ. In contrast to this picture, there are large numbers of short, widely dilated, anastomosing capillaries in the main body of squamous-cell carcinomas (fig. 13), while in malignant melanoma, the vessels seem to be intermingled with the mass of tumor cells, unaccompanied by any significant amount of connective tissue (fig. 14). In the tissue surrounding both tumors there are increased numbers of altered capillaries similar to those seen around the basal-cell carcinomas.

ANATOMY OF SKIN CAPILLARIES OF HUMAN PREMALIGNANT LESIONS

Senile keratosis and Bowen's disease have long been considered as premalignant disorders. Some authors, with considerable justification, classify them as carcinoma *in situ* (*28*). The corium immediately below the epidermal changes of senile keratosis and Bowen's disease shows most striking vascular abnormalities. There is great elongation of the capillary limbs, tortuosity, dilation, gross shunting, and an absolute increase in the number of vessels. Each papilla contains several capillaries, with markedly dilated, deformed, and convoluted tops, resembling a tangled skein (fig. 15). Where invasion of the dermis becomes evident, these vessels take on the shape of blunted sinusoids that anastomose in circles (fig. 16). The vessel changes begin sharply at the point where epithelial hyperplasia begins and are most evident under areas of major epithelial atypism or anaplasia (figs. 17 and 18).

It is of interest that the described vascular pattern is almost entirely absent from the very hyperplastic changes of keratoacanthoma and that the nodules of Kaposi's sarcoma also show few capillaries.

The changes noted in vessels in the premalignant lesions correspond well to the observations of Dreifus and Bloch (*29*) and Kreyberg (*30*) in mouse skin painted with carcinogens, and the tumor capillaries seen in squamous-cell carcinoma and melanoma are similar to the description of the vascular anatomy of transplanted animal neoplasm (*7*, *12–14*).

PHYSIOLOGIC OBSERVATIONS

Physiologic Responses of Tumor Blood Vessels

True capillaires have tone but are not actively contractile, the diameter of their lumen being determined mainly by changes in the precapillary vessels (*22*). No studies on the effect of adrenalin or atropine on the capillaries of tumors are available. Ergot and pitressin, which cause a blanching of the entire animal, have no effect on the tumor (*31*). A series of studies have been made on the effect of hypotension, produced by a variety of pharmacological agents, on tumor blood vessels. Algire and associates (*32–35*) report that hypotension, however produced, causes a rapid collapse and emptying of most tumor blood vessels. This phenomenon is demonstrable even with a reduction of arterial blood pressure that has no clinical effects on the experimental animals. This is apparently due to a rapid collapse of the thin-walled vessels that are under the continuous pressure of the tumor mass. Histamine, pitressin, *Serratia marcescens* polysaccharide, and podophyllin alkaloids have such an effect.

Although there is a general relationship between the reduction in tumor blood flow and the magnitude and duration of the drop in blood pressure, prolonged and intense anoxia (more than 2 hr) is required before significant necrosis of neoplasms occurs (*35*). In this sense, the tumor tissue is highly resistant to the effects of diminished blood flow.

The few studies of tumor capillary permeability on record have been performed with colored dyes. After the administration of isamine blue (*36*, *37*) and fluorescein (*38*), the dyes appear rapidly in malignant neoplasms, stain the tissue more intensely than normal control tissues, and tend to persist longer. On this basis it has been suggested that blood flow in tumors is more rapid than in normal tissues, and that incomplete or inadequate differentiation of the endothelial cells increases capillary permeability (*38*).

Blood Flow and Oxygen Transport in Tumor Vessels

The disorderly nature of the blood-vessel network in tumors has precluded direct studies on the rate of blood flow. The temperature in the tissue overlying the tumor masses is increased, patent blood vessels are numerous, and the oxygen content in the veins draining the tumor bed is very high. Some investigators have assumed that blood flow through malignant lesions is rapid and tissue oxygen consumption very low (*39*, *40*). A re-examination of existing data reveals the following:

1) In general, neoplasms contain a rich network of anastomosing thin-

walled, wide, sinusoidal capillaries; the tumor mass is surrounded by large numbers of dilated, coronal arteries and veins.

2) Dyes and radiopaque materials, though appearing very rapidly in the tumor vessels, tend to persist in these channels much longer than in normal tissues.

3) Although the temperature of the tissue adjacent to the tumor is usually significantly higher than in surrounding areas, the main body of the neoplastic mass has a temperature lower than normal.

4) The large, efferent tumor vessels have oxygen levels that often approach those of arterial blood and are significantly higher than those in nearby veins. At the same time, direct measurements of tissue oxygen tension and content of glucose and lactic acid in tumor capillaries would indicate almost complete removal of capillary oxygen in the main body of the malignant tissue.

While these divergent findings appear to be incompatible, it is possible to achieve some theoretical reconciliation when one considers the known structural characteristics of the tumor-vessel bed.

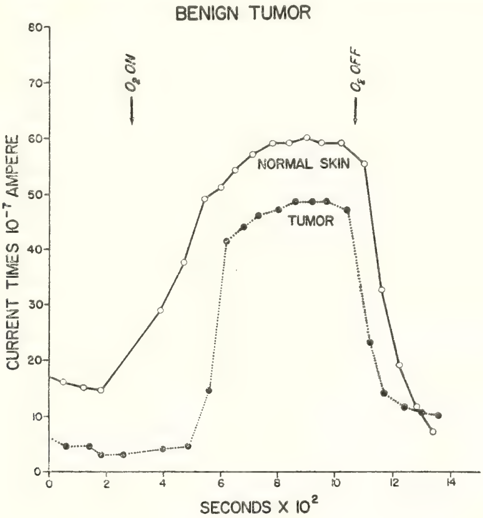
Rapid blood flow through the many dilated and coiled arteries surrounding the edge of the tumor elevates the tissue temperature around the neoplasm. A significant amount of arterial blood is shunted directly to the large draining veins without passing through the tumor bed. This accounts for the rapid filling of veins observed in angiograms and the low difference in arteriovenous oxygen. Blood, not shunted around the periphery, enters the true tumor circulation where the huge increase in the total cross-sectional area markedly decreases the rate of flow through the capillaries. This slow, often circular blood flow within the tumor accounts for the puddling and long persistence of radiopaque dyes and the lower temperature of the tumor. Prolonged exposure of capillary blood to metabolizing tissue explains the great deoxygenation and lactic acid accumulation, without the necessity to postulate a low oxygen consumption of tumor tissue. This hypothesis receives indirect support from the studies of the measurements *in vivo* of oxygen tension of tumor tissue.

POLAROGRAPHIC STUDIES

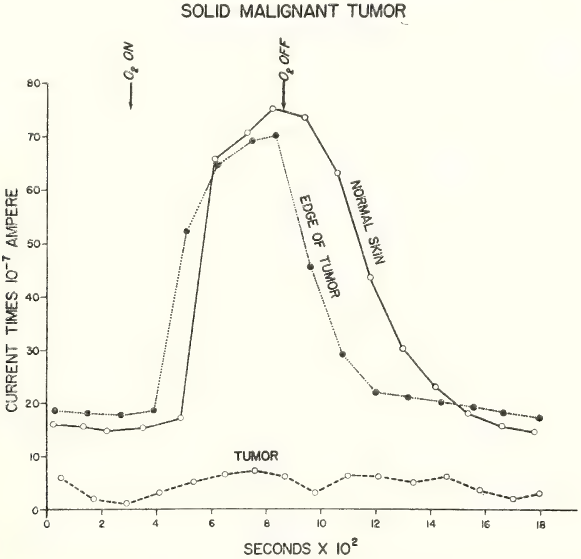
Early investigations on oxygen tension of tumor tissue *in vivo* suggest the existence of an abnormally low tissue p O_2 in a variety of primary and metastatic cutaneous tumors (*41*). It was shown that it is possible to measure relative mean tissue oxygen tension by a polarographic technique utilizing stationary platinum oxygen cathodes (*41*). The polarization current (P.C.) obtained from such electrodes reflects mean tissue p O_2, though quantitative measurements have not yet been made with great accuracy.

By combining stationary oxygen micropolarography with a sort of "oxygen tolerance test," Urbach and Noell (*41*) showed that: 1) The P.C. of a variety of malignant skin neoplasms was significantly lower than that of the surrounding normal skin, and 2) there was little or no increase of tissue P.C. in response to oxygen breathing in solid cancers, while a normal or near normal response was obtained in inflammatory lesions, benign neoplasms, and lymphomas; malignant melanoma occupied an intermediate position (text-figs. 1, 2, and 3, and table 1).

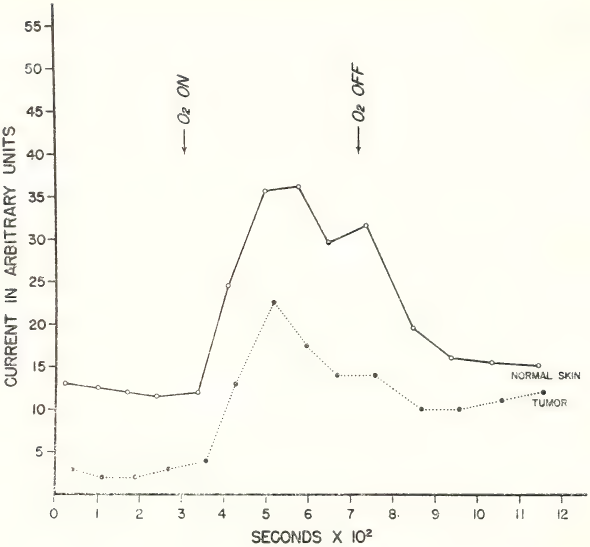
The 2 possible physiologic causes that may prevent a significant rise in oxygen tension of tumor tissue during oxygen breathing can be described



TEXT-FIGURE 1.—Response of polarization current of benign cellular nevus to inhalation of 100 percent oxygen [Urbach and Noell (*41*)].



TEXT-FIGURE 2.—Response of polarization current of squamous-cell carcinoma and of adjacent normal skin to inhalation of 100 percent oxygen [Urbach (*42*)].



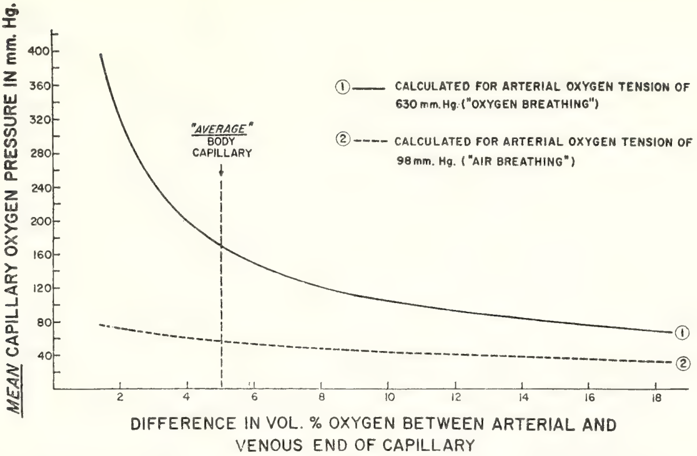
TEXT-FIGURE 3.—Response of polarization current of metastatic malignant melanoma nodule and adjacent normal skin to inhalation of 100 percent oxygen [Urbach (*42*)].

TABLE 1.—Response to O_2 inhalation of polarization currents (P.C.) obtained from: malignant tumors, benign tumors, scars, premalignant lesions, and adjacent normal skin [Urbach and Noell (*41*)]

	Number of observations	P.C. during air breathing ×10^{-7} amp	P.C. during O_2 breathing ×10^{-7} amp	P.C. O_2 P.C. air ×10^{-7} amp
Malignant tumors				
Normal skin	43	20.5 ± 6.64	64.4 ± 18.9	41.0 ± 6.7*
Carcinoma and sarcoma	32	10.0 ± 4.6	15.4 ± 13.6	5.1 ± 3.8
Melanoma	6	3.2 ± 0.34	39.0 ± 31.6	23.0 ± 4.6*
Lymphoma	5	9.2 ± 9.0	70.8 ± 26.4	63.0 ± 19.4*
Benign tumors				
Normal skin	16	22.1 ± 5.3	64.4 ± 15.0	42.2 ± 7.2*
Nevi xanthomas	7	10.3 ± 6.0	40.1 ± 20.4	31.2 ± 10.2*
Scars	4	15.5 ± 3.0	28.5 ± 8.6	14.2 ± 4.3†
Keratoses	5	15.6 ± 3.6	61.0 ± 12.2	44.0 ± 12.4*

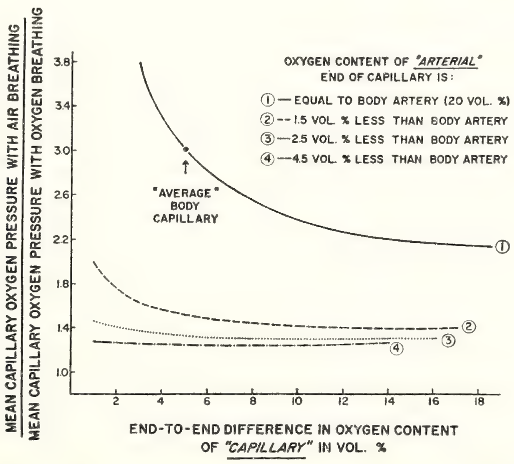
*Significant at better than 0.001.
†Significant at better than 0.01.

as follows: 1) The oxygen consumption of carcinomas may change with oxygen tension. The best argument against this possibility is that the P.C. in malignant melanomas and malignant lymphomas increased in response to oxygen breathing, similar to that in normal skin. There are no known differences in metabolism between these malignancies and those of the carcinoma-sarcoma type; and 2) the vascularization of carcinomatous tissue may be predominantly venous. As illustrated in text-figures 4 and 5, only a small rise in oxygen tension would occur



TEXT-FIGURE 4.—*Mean* oxygen pressure of capillary [Urbach and Noell (*41*)].

with oxygen breathing if the blood, reaching carcinomatous tissue, is not as loaded with oxygen as the arterial blood. For instance, if the blood reaching the point of measurement was already depleted by 2.5 volumes-percent of oxygen, the mean oxygen tension of the tumor-capillary bed would rise only by 40 percent as these capillaries deliver 10 volumes-percent of oxygen to the cancer tissue. Thus, if the blood vessels, which have grown into the tumor, are thin walled and have a slow blood flow, oxygen would be lost as the blood moves to major sites of exchange within the tissue. Such abnormalities of vascularization have been demonstrated in carcinomas and sarcomas, but not in lymphomas and melanomas. Thus, the marked anatomical differences in the blood supply of malignant tumors provide an explanation for the observation that carcinomas and sarcomas lack a significant rise in oxygen tension in



TEXT-FIGURE 5.—Increase of *mean* oxygen pressure of capillary with change from air to oxygen breathing [Urbach and Noell (*41*)].

response to oxygen breathing; the "venous" character of their circulation prevents such an increase.

Some Implications of Tumor-Vessel Abnormalities

In 1924, Warburg (*43*) and his co-workers stated that "When we consider that all tissue at rest has some slight glycolytic activity, nothing prevents us from the assumption that there exists an unequal distribution of glycolysis, and that some cells have the embryonic type, but the majority show no glycolytic activity. If anoxia acts on such a cell mixture (by pressure, vessel sclerosis, presence of bacteria, etc.) then those cells lacking the ability of anaerobic glycolysis must die; those having it may survive. We could assume that some of the latter actually survive, and thus are able to utilize the energy derived from anaerobic glycolysis, and grow because of this. Thus if anoxia becomes chronic, tissues with the anaerobic glycolytic capabilities of embryonal cells develop. Since it has been grown under anaerobic conditions, it will have little respiration. As a matter of fact, we know from experiments with embryos that anoxia injures respiration.

"This theory replaces the indeterminate 'irritation' with the definite 'anoxia.' Accordingly, tumors develop not from misplaced embryonal rests, but from differentiated growing cells, normally occurring in all tissues. Since anoxia will kill all cells unable to glycolyze, the total glycolytic activity of the new tissue will rise, but not above the glycolytic activity of the remaining cells.

"Why growing tissue, irrespective of its methods of origin grows so uncontrollably and irregularly, proportionate to the disturbance in the relationship between glycolytic splitting and oxidation remains a mystery."

Since Warburg's early work, giant strides have been made in the understanding of metabolic pathways, the submicroscopic anatomy of cells, and the biology of cancer tissue. The brief review of our knowledge of tumor blood vessels, their origin, growth, and response that has been presented here may be interpreted in various ways. Several facts, however, seem paramount. Solid tumors have a rich and disordered vasculature, consisting predominantly of wide, sinusoidal capillaries. In transplanted tumors the vasculature is derived primarily from the host tissue, though apparently under some influence of the implanted tumor. Agents known to be carcinogenic induce in the host tissue more or less specific vessel changes resulting in impairment of the circulation and chronic anoxia. Anatomical considerations, indirect studies, and direct measurements of oxygen tension all testify to the marked tissue anoxia, combined probably with a deprivation of other nutrients in the main malignant tumor mass. It appears, as observed by Haddow (*44*), that "Cancer cells arise and commence their career of proliferation under conditions which impair the life of normal cells."

Without discounting the importance of genetic, viral, and other influences, one can see that evidence strongly points to anoxia as a major culprit in carcinogenesis. Foulds (*45*), Berenblum and Shubik (*46*), and Rush (*47*) have proposed that cancer is the result of a multistage process, beginning with a genetic change in a cell and continuing with the growth of this potential cancer cell in the proper environment.

In a previous publication the author (*42*) proposed, with some hesitation and drawing considerably on previously published work, a composite working hypothesis that seems to fit a considerable amount of the data known at this time about carcinogenesis: "A cell in a given tissue, under the influence of some stimulus (ionizing radiation, chemical carcinogen, aging, anoxia, antimetabolites, viruses, etc.) undergoes genetic alterations that result in altered metabolic capabilities. If such metabolic alterations confer a distinct survival value upon this cell, as compared to its neighbors, and such a 'potential cancer cell' finds itself in an environment that favors its acquired metabolic alteration, but interferes with the survival of 'normal' neighbors, then, the 'tumor' cell would have a biological advantage.

"If we substitute 'increased glycolysis' for 'altered metabolism' and 'anoxia' for 'favorable environment,' perhaps one of the reasons for unbridled growth characteristics of cancer is the capability, once a certain critical mass has been established, to tailor its environment to its needs. Certainly, the grossly abnormal circulation, characteristic of solid tumors, must and does result in tissue hypoxia. As Algire and others (*12–16*) have shown, even a small piece of organized tumor can, on transplantation to a compatible host, reproduce this peculiar vessel structure in an otherwise normal tissue."

I suspect that a casual statement made by Professor Warburg in a recent letter (*48*) will be validated: "Pasteur's 'La Vie Sans Air' ist der Schlüssel zum Krebsproblem."

Finally, the gross abnormalities of the blood vessel system of the tumor which seem to be present in most malignant tissue result in a considerable slowing of blood flow in the main body of tumors. Since the publication of our work on tumor oxygen tension, Gullino (*49, 50*), in a series of ingenious experiments, has confirmed this phenomenon. He and his associates showed that tumor blood flow, measured directly and indirectly, is of an order of magnitude of about 1.5 cc per 100 g of wet tissue per minute, or about one tenth to one twentieth that of such epithelial organs as kidney, ovary, or liver. This slow blood flow has some very real and as yet neglected implications in cancer chemotherapy.

The emphasis in this field has been on the development of more active and more specific chemical agents. But newer and better chemicals will not have much success if one cannot get them to the tumor cells. Further confirmation of the importance of this problem comes from the dramatic differences in cytotoxic effects obtained by direct injection of chemotherapeutic agents into tumors (*51*) as compared to the slow or even indifferent effect of the same compounds when injected intravenously or given by some other systemic route.

It thus appears to me that only when methods are devised to circumvent the anatomical road block, posed by the peculiar properties of the tumor circulation, will the chemotherapy of cancer become truly effective.

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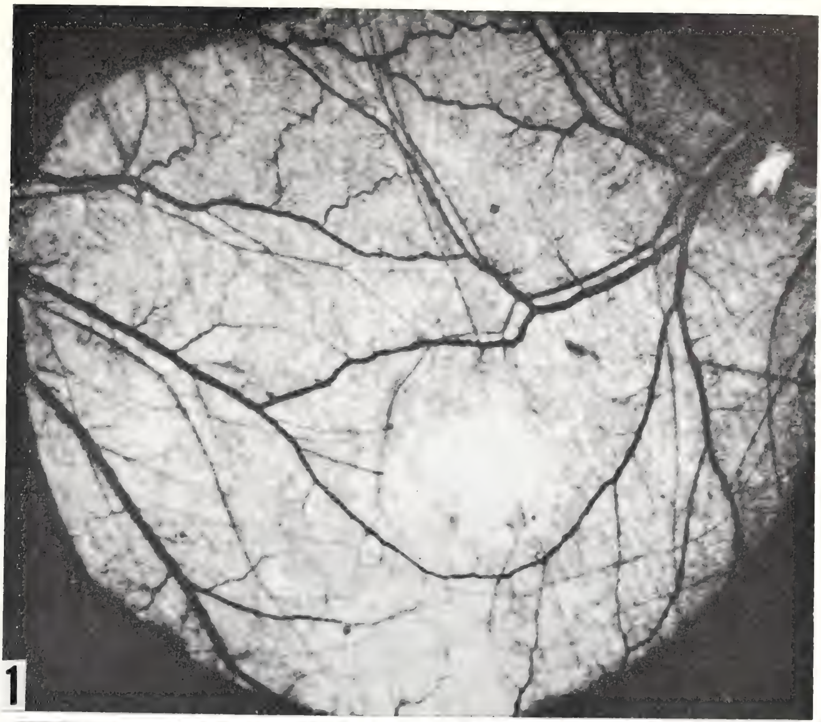
Plate 59

Figure 1.—Subcutaneous tissue within a transparent chamber in the mouse, showing normal vascular supply of connective tissue and subsiding implant reaction [Algire and Chalkley (*12*)].

Figure 2.—Fully developed vascular bed of a mouse mammary carcinoma on the 17th day, showing irregular pattern and variation in size of vessels. *Note* particularly huge marginal vessels and blood-filled cul-de-sacs [Algire and Chalkley (*12*)].



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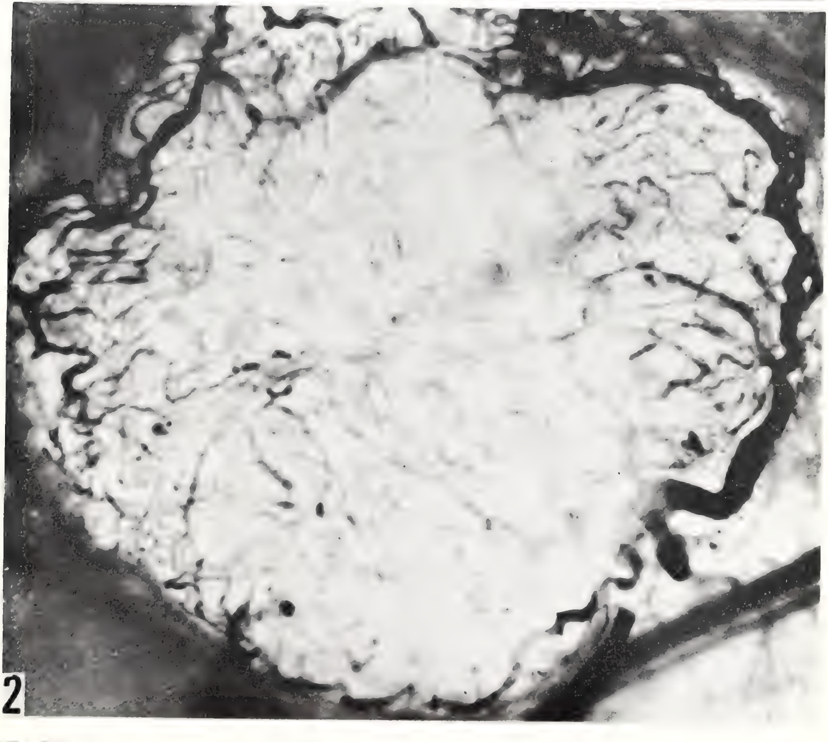

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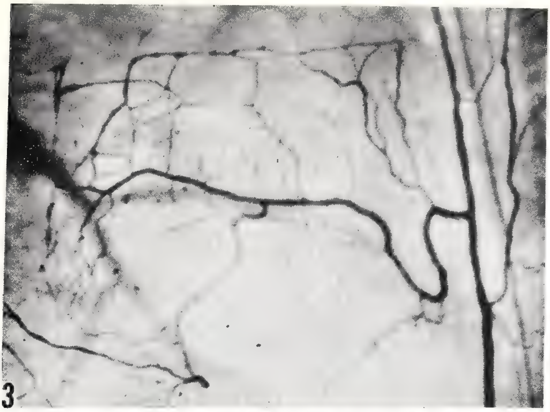
Plate 60

Figure 3.—Normal-appearing vascular bed at site of implant of lymphoma. Tumor mass fills most of the field, but it is only indistinctly visible. Anatomy of vessels normal. (Algire, personal communication.)

Figure 4.—Liver specimen with metastatic tumor, injected with lead-turpentine mixture in arterial tree. Good demonstration of abnormal caliber and arrangement of tumor vessels. (Courtesy of Dr. E. Lasser and the Department of Radiology, Roswell Park Memorial Institute, Buffalo, N.Y.)

Figure 5.—Osteogenic sarcoma. Early arterial phase angiogram shows rapid filling of abnormal vessels in tumor mass and rapid passage of contrast medium into dilated veins. (Courtesy of Dr. E. Lasser and the Department of Radiology, Roswell Park Memorial Institute, Buffalo, N.Y.)

Figure 6.—Osteogenic sarcoma. Late venous phase angiogram showing increased number and coiling of vessels, persistent visualization of cul-de-sacs and enlarged veins surrounding tumor. (Courtesy of Dr. E. Lasser and the Department of Radiology, Roswell Park Memorial Institute, Buffalo, N.Y.)



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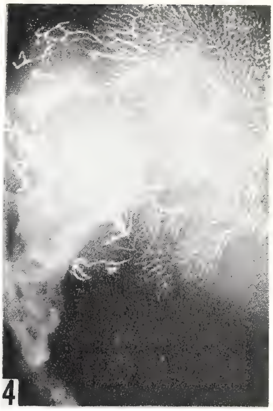

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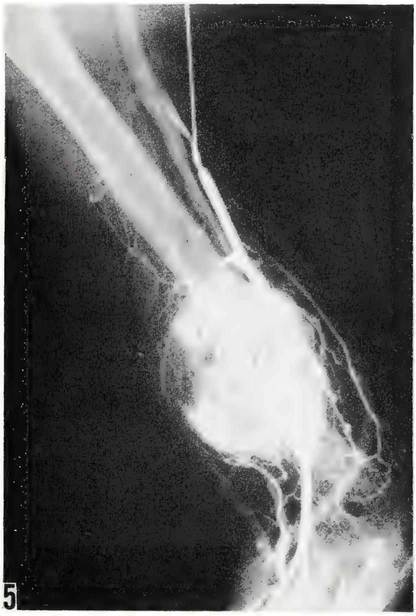

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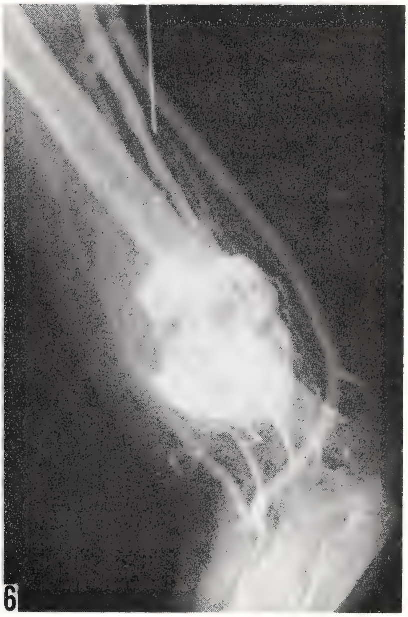

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Plate 61

Figure 7.—Alkaline phosphatase stain of capillaries in normal human skin.

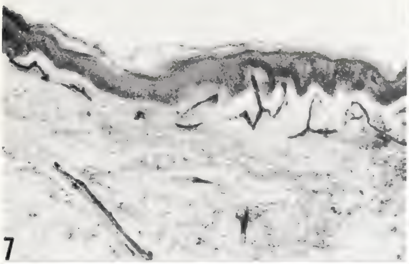
Figure 8.—Section through a dermal cellular nevus. *Note* slightly elongated but otherwise normal capillaries in between masses of nevus cells. Alkaline phosphatase stain. × 53

Figure 9.—Acute vesicle of herpes simplex. *Note* paucity of capillaries that appear essentially normal in spite of severe inflammatory process. Alkaline phosphatase stain. × 53

Figure 10.—Horizontal section through a nodule of molluscum contagiosum. Capillaries in central stalk and in corona are distinct. No vessels are seen in area affected by virus. Alkaline phosphatase stain. × 53

Figure 11.—Horizontal section at the papillary level through a papule of psoriasis. *Note* regular arrangement of papillae and presence of an ascending and a descending capillary loop in each papilla. Alkaline phosphatase stain. × 53

Figure 12.—Section through a basal-cell carcinoma. *Note* increased number of capillaries, marked elongation, branching and cross anastomoses. Capillaries are not seen in tumor mass itself. Alkaline phosphatase stain. × 53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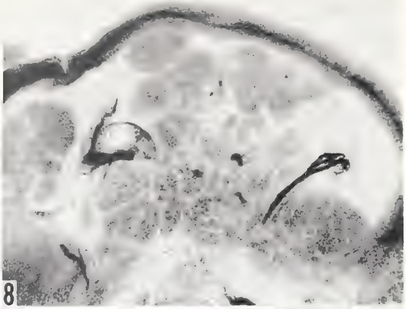
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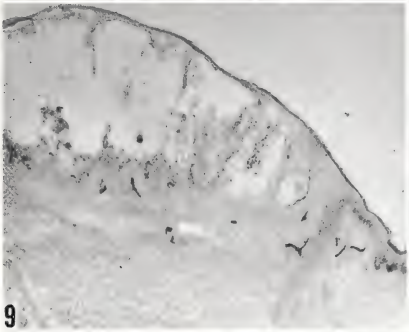
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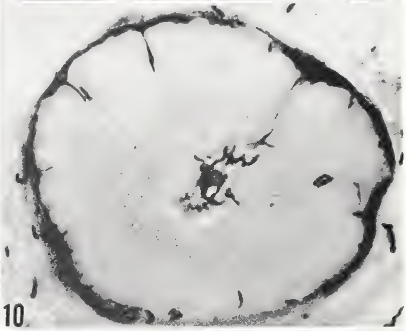
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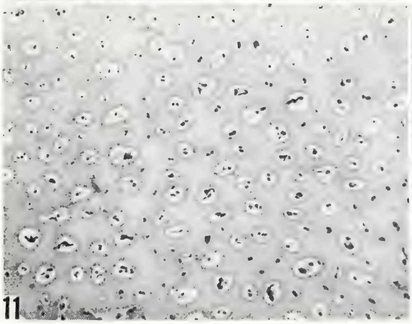
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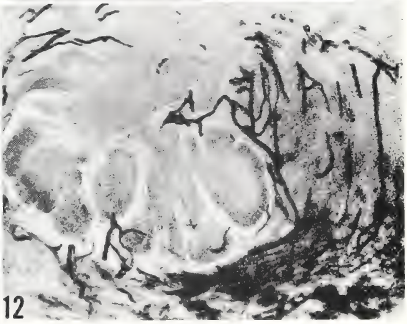
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PLATE 62

FIGURE 13.—Horizontal section through a nodule of anaplastic squamous-cell carcinoma. *Note* widely dilated capillaries that cross anastomose to make circular channels. Capillaries seem to lie between tumor masses and are not accompanied by significant amounts of connective tissue. Alkaline phosphatase stain. × 53

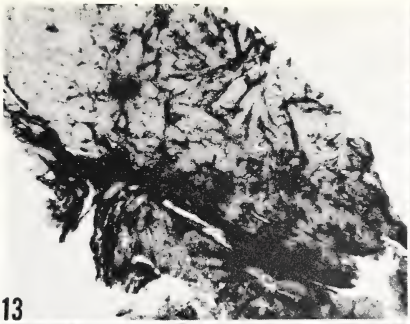
FIGURE 14.—Section through a metastatic nodule of malignant melanoma. *Note* long, thin capillaries arranged in parallel rows. Capillaries apparently lie free among tumor cells. Alkaline phosphatase stain. × 53

FIGURE 15.—Senile keratosis with invasion as squamous-cell carcinoma. *Note* marked increase in capillaries, elongation and tortuosity, and several capillary loops in each papilla. There is great abnormality of tops of capillaries that surround invading tumor. Alkaline phosphatase stain. × 53

FIGURE 16.—Capillaries surrounding the down-growing pegs of squamous-cell carcinoma arising in a senile keratosis. *Note* large number of capillaries with marked dilatation, and appearance beginning to simulate that seen in anaplastic squamous-cell carcinoma. Alkaline phosphatase stain. × 75

FIGURE 17.—Section from border of a lesion of Bowen's disease. *Note* marked increase in capillaries, several capillary loops in each papilla, marked distortion of top of capillary forming a ball-like appearance, and cross anastomoses. Epidermis shows only minor changes at this point. Alkaline phosphatase stain. × 53

FIGURE 18.—Section from middle of a lesion of Bowen's disease. Epidermis is hyperplastic, but there is no breakthrough as yet. Capillaries are increased in number, elongated, with a "skein of wool" appearance and tortuosity and cross anastomoses of the top. Alkaline phosphatase stain. × 53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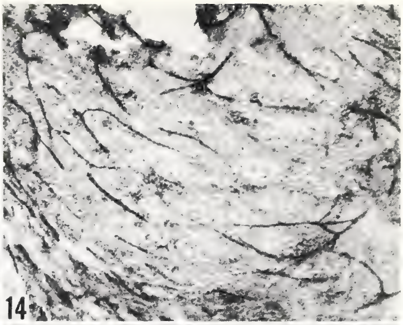
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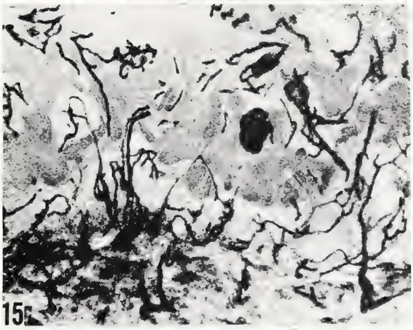
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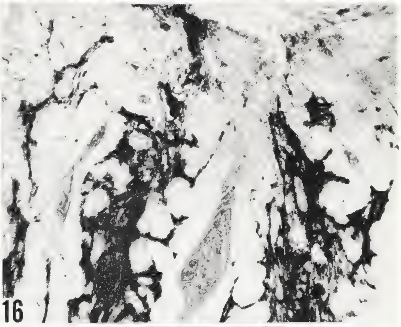
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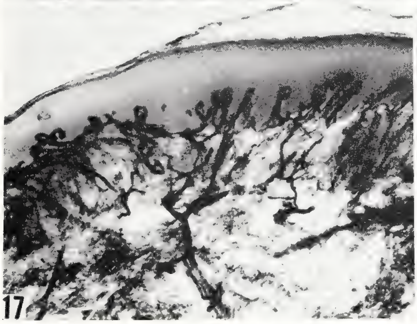
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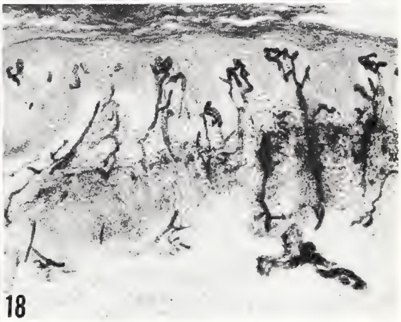
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THE ROLE OF MELANOCYTES IN SKIN CARCINOGENESIS[1, 2]

WALTER C. QUEVEDO, JR.,[3] Biology Department, Brown University, Providence, Rhode Island

SUMMARY

Pigmented skin tumors may result when melanocytes participate in carcinogenesis. Melanocytes form the essential units of structure of malignant melanoma and may be included as secondary ingredients in several different types of integumentary tumors. The role of melanocytes in the process of tumorigenesis is discussed in the light of recent findings on melanocyte form and function. The functional integration of melanocytes with the activities of the skin as a whole is stressed, particularly as it bears on melanocyte neoplasia. The histogenesis of "spontaneous" malignant melanoma is briefly described and compared to that of carcinogen-induced, primary melanocytic tumors. It is shown that certain melanocytic tumors may arise from melanocytes at the dermoepidermal junction, in the dermis, and hair follicles. The mechanisms which trigger the malignant transformation of melanocytes are obscure, though radiations, mechanical trauma, chemical carcinogens, and hormonal and genetic factors have been indicated to be of significance in this process. Attempts to relate the gross pigmentation of primary and secondary melanocytic tumors to their potential malignancy have met with controversy. Similarly, the importance of skin and hair coloration in skin carcinogenesis remains unsettled. On the basis of accumulating evidence for a symbiotic relationship of epidermal cells and melanocytes, it is speculated that the responses of melanocytes to carcinogens may be indirect, being dependent on appropriate cues from cells of other lineage.—NCI Monogr. No. 10: 561–575, 1963.

MELANOCYTES ARE characteristic components of mammalian skin and normally synthesize melanin pigments in close harmony with the other integumentary functions. Melanocytes may also play primary and secondary roles during carcinogenesis in mammalian skin. It is generally agreed that they form the essential units of structure in malig-

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

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[3] The author wishes to thank Dr. William Montagna of Brown University and Dr. George Szabó of Harvard Medical School for reading the manuscript and for their helpful suggestions, and also Dr. Alfred W. Kopf of the New York University Post-Graduate Medical School for making available a manuscript now in press.

nant melanoma and the secondary ingredients of certain melanotic tumors derived primarily from other cellular components of the integument. Although melanotic skin lesions have been studied extensively in man and experimental animals, more is known about the mechanisms that regulate normal melanocyte function than those which enmesh melanocytes in the process of carcinogenesis. This paper will stress our current knowledge of this problem from the viewpont of: 1) the normal melanocyte—its anatomical distribution, reproduction, and integration with skin functions; 2) the histogenesis of primary melanocytic tumors, *e.g.*, malignant melanoma; 3) the relationship of melanocytes to integumentary tumors arising from other cell types; and 4) cellular interactions during carcinogenesis which involve melanocytes.

MAMMALIAN MELANOCYTES—GENERAL CONSIDERATIONS

A number of review papers published recently provide a valuable guide to the extensive literature dealing with the form and function of mammalian melanocytes (*1–8*). Only a brief account can be presented here.[4]

Melanocytes are unicellular secretory glands which synthesize a pigmented granular product of melanoprotein (*1*). Embryologically, they are derived from the neural crest (*2*). At an early stage of development, presumptive melanocytes (melanoblasts) invade the embryonic skin, at first the presumptive dermis and later the epidermis and its derivatives (*9*). In adult mammals, integumentary melanocytes may be situated at the dermoepidermal junction, in the dermis, and associated with pilosebaceous units and sweat glands (*1*). Melanocytes of the dermoepidermal junction and hair bulbs regularly release melanin granules to surrounding epidermal cells. This property of melanocytes, termed "cytocrine activity" by Masson (*10*), contributes to the characteristic coloration of the skin and hair. Melanin granules may be yellow, brown, or black (*5*). The type of melanin synthesized by melanocytes is specified by genetic mechanisms that act either directly within melanocytes or indirectly through their cellular environment (*11*). Melanin synthesis begins with the tyrosinase-catalyzed oxidation of tyrosine to dihydroxyphenylalanine (dopa) which in turn undergoes stepwise conversion to melanin (*8*). Fitzpatrick and coworkers (*8*), on the basis of careful biochemical and morphologic studies, have emphasized that melanin synthesis must be regarded as the evolution of a complex cytoplasmic particle, the melanosome. The degree of melanization of melanosomes is inversely proportional to their tyrosinase activity (*8*). The intracellular origin of melanosomes is still a matter of debate; some investigators favor an origin from mitochondria (*12*) and others stress an origin unrelated to that of mitochondria, but involving the ribosomes and Golgi apparatus of melanocytes (*13–15*). The electron microscope has provided important information about the fine structure and maturation of melanosomes (*16, 17*). The mechanism by means of which transfer of melanin granules to epidermal cells is accomplished (cytocrine activity) remains unsettled, but is receiving careful attention by electron microscopists (*16, 18*).

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[4] To keep bibliography within manageable limits, review papers are cited wherever possible. Primary references for work cited in this manner may be found within these publications.

Melanocyte populations have been studied extensively in the epidermis of human skin (*1, 19*). It is clear that epidermal melanocytes are not uniformly distributed throughout the body integument, but show marked regional variations both in numbers and structure. They are most numerous in the epidermis of the head, 1,930 ± 60 per mm^2, and genitalia, 2,380 ± 280 per mm^2, and least numerous in that of the trunk, 890 ± 70 per mm^2 (*19*). Although showing marked regional differences in population, melanocytes are symmetrically distributed throughout the body, being about equal in number within contralateral anatomical sites of the same individual. Racial or sexual differences in melanocyte populations are of no significance in man; consequently, variations in color of human skin do not reflect differences in melanocyte density. Instead, they are related to the quantity and/or color of melanin granules elaborated by physiologically different melanocytes (*19*).

Although direct experimental proof is lacking, much circumstantial evidence indicates that epidermal melanocyte populations are not static but undergo turnover by mitotic division of resident melanocytes (*1*). The cells of Langerhans, nonpigmented dendritic cells found at superficial levels in the epidermis, have been described as desquamating "effete" melanocytes (*1*). However, not everyone agrees with this interpretation (*1*). Recently, Birbeck *et al.* (*20*) found striking similarities between melanocytes and Langerhans' cells in human skin examined under the electron microscope. Indirect evidence that melanocytes may be lost through the epidermis is provided by studies with thorium X (*21*). After application of this agent to human skin, pigmented (dopa-positive) melanocytes are found at high levels in the epidermis, *i.e.*, at the same sites where Langerhans' cells are normally observed. Thorium X treatment thus results in the dislodgment of mature pigment-producing melanocytes from the basal layer of the epidermis and their subsequent migration into more superficial regions.

Biochemical and histochemical studies have demonstrated marked changes in specific metabolic processes within skin that parallel the cyclic growth and quiescence of hair follicles (*6*). The responses of the skin to X rays and chemical carcinogens are particularly influenced by "hair growth cycles" (*22*). Epilation of hair by X rays is most readily accomplished at times of follicular growth (*22*). Chemical carcinogens produce greatest skin damage and highest tumor yields if applied when hair follicles are quiescent (*22, 23*).

Changes in the morphogenetic activity of hair follicles are also reflected in the cyclic variations in melanocyte physiology. If quiescent hair follicles of pigmented mice are exposed to 1000 r of X rays, approximately 80 to 90 percent of the follicular melanocyte population is destroyed, as indicated by the regrowth of hairs which are totally white or partly depigmented (mosaics) (*24*). However, significantly fewer depigmented hairs are recovered for a given dose of X rays when growing hair follicles are irradiated. Thus, the admininstration of 1000 r of X rays during a period of follicular growth results in only approximately 50 percent "graying" (white and mosaic hairs) in the next hair generation (*24*). Weissenfels (*25*) has described the degeneration of melanocytes after X irradiation.

Additional evidence for a close relationship of melanocyte function and hair growth cycles is obtained in the response of melanocytes to chemical carcinogens (*26*). After a single topical application of methylcholanthrene to the skin of pigmented rabbits, many normally nonpigmented melanocytes of the dermoepidermal junction and upper external root sheaths of hair follicles become melanogenically active. Melanogenesis occurs earliest when the carcinogen is applied during periods of hair growth. It would appear that the nonpigmented melanocytes are most responsive to activation at a time when the melanocytes of the hair bulbs normally produce pigment. Since there is less manifest damage done to epidermal cells by methylcholanthrene during periods of follicular growth, it is also possible that melanocyte activation is linked to the altered metabolism of epidermal cells. Although the mechanisms involved in triggering the participation of melanocytes in carcinogenesis are still obscure, these studies suggest *that the induction of malignant changes by extrinsic or intrinsic factors involves melanocytes, not as isolated "targets," but as members of a complex and changing cellular community.*

PRIMARY MELANOCYTIC TUMORS

Human Melanoma

Although there is not complete agreement, malignant melanoma in man is generally considered to arise from the neoplastic transformation of melanocytes situated at the dermoepidermal junction (*27, 28*). Estimates range from 18 to 83 percent on the number of cases of malignant melanoma traceable to an origin within pre-existing pigmented nevi (*29*). Pack (*30*) reported a similar, but not identical, frequency distribution of pigmented nevi and malignant melanoma in human skin. Szabó (*19*) subsequently compiled a frequency distribution for melanoma based on a compilation of 3,909 cases reported in the literature. After a careful comparison of the frequency distribution of melanoma with that of melanocytes, nevi, and skin appendages (hair follicles and sweat glands), Szabó found the distribution of malignant melanoma most closely related to the areas with the largest numbers of hair follicles and sweat glands.

Few agree on the cellular composition of pigmented nevi. It has been stated that pigmented nevi are derived from melanocytes (*31*), melanocytes and Schwann cells (*32*), and epidermal cells (*33*). Most of us agree that malignant melanoma may derive from melanocytes, but the role of nevus cells in the origin of this lesion is still unsettled (*29*, *34*). Becker has maintained that nevus cells (fig. 1) do not give rise to malignant melanoma (*35*). This view stresses the importance of melanocytes contained within nevi as the sensitive elements. Mishima (*36*), in a study of *Melanosis circumscripta praecancerosa* (Dubreuilh), concluded that malignant melanoma may originate from epidermal melanocytes without nevus-cell formation (fig. 2). It appears that malignant melanoma arises only within the so-called "junctional" and "compound" nevi; with the rarest of exceptions, strictly "intradermal" nevi do not become malignant (*29*). Significantly, the melanocytic nature of the "intradermal" nevus cell recently has been questioned by Winkelmann (*37*).

Invasive malignant melanoma in man is characterized by a proliferation of cells at the dermoepidermal junction (*34*). Primary tumors may be distinguished from metastases on the basis of this "junctional" activity. As indicated, there is now little doubt that this tumor may result from the malignant neoplasia of melanocytes. The role of nevus cells in the formation of malignant melanoma remains to be clarified. Histological studies have revealed nevus cells in 23 to 100 percent of the total number of cases of malignant melanoma examined (*29*). In its earliest stages, it is difficult to distinguish malignant melanoma from the simple activation of a junctional nevus (*28*). However, as development progresses the tumor cells become vacuolated, contain variable amounts of melanin, and their nuclei are irregularly shaped (fig. 3). The tumor cells, round, oval, or fusiform, stream downward into the dermis. The cells lack cohesiveness and tend to dissociate from the primary tumor (*38*). They may also invade the upper layers of the epidermis. The architecture of the undersurface of the epidermis is modified in advanced melanoma (figs. 4 and 5). In early stages of tumorigenesis, an inflammatory infiltrate and large numbers of macrophages may be present in the upper dermis. This inflammatory response may disappear in advanced melanomas (*34*). The melanin content of melanomas varies greatly, with some tumors heavily melanized and others virtually amelanotic. Metastases may take place through the lymphatics and blood stream with liver, lungs, brain, and skin the primary metastatic targets (*34*).

Virtually nothing is known about the mechanisms that bring about the transformation of melanocytes into melanoma cells. Mechanical trauma has been stated to elicit malignant melanoma within nevi and even within normal skin. Some claim that all trauma is potentially dangerous; others maintain that there is no evidence that a single injury to a nevus precipitates the onset of a malignancy (*28*, *39*). However, there appears to be less disagreement over the possibility that chronic irritation or frequent traumatizing of junctional nevi may bring about malignant transformation (*28*).

Malignant melanoma occurs with high frequency in the head and neck, regions of the body normally exposed to sunlight. Malignant melanoma seems to occur most frequently in human beings of fair complexion (*28, 30*). Lancaster concluded that the incidence of human melanoma is highest in geographical locations receiving intense solar (ultraviolet) irradiation (*40, 41*). Thomson (*42*) compared the transmission of ultraviolet light by layers of stratum corneum obtained from the hip (unexposed) regions of Caucasians and Negroes. The greater opacity of the stratum corneum of the Negro to ultraviolet light was attributed to its greater content of melanin pigment. In contrast to other workers, Macdonald failed to find evidence for racial differences in the incidence of melanoma or any correlation with exposure to sunlight (*43*, and its appended discussion). The occurrence of malignant melanoma in normally clothed regions of the body clearly indicates that factors other than solar radiation must also be effective in eliciting the production of this tumor type. In addition to radiations and mechanical trauma, chemical carcinogens may also induce malignant transformation within melanocytes (*44*). Internal mechanisms, hormonal and/or genetic in nature, appear to influence this process (*44*). There is little doubt that a variety of stimuli of internal or external origin is capable of triggering the onset of melanoma formation in man; however, important details as to the early events in tumor induction are lacking. Certain findings from studies of experimental carcinogenesis in laboratory animals have some bearing upon this important problem.

Experimental Induction of Melanotic Tumors

Studies of carcinogenesis in rodents indicate that primary melanocytic tumors may arise from melanocytes of the dermoepidermal junction as well as from those situated in, or derived from, the dermis, hair bulbs, and connective tissue sheaths of hair follicles (*45–48*).

Melanotic lesions, including malignant melanomas, have been elicited in the skin of a variety of experimental animals following cutaneous applications or injections of chemical carcinogens (*49–55*). When Burgoyne *et al.* (*45*) painted pigmented mice repeatedly with 7,8,12-trimethylbenz[*a*]anthracene, blue nevus-like foci developed entirely within the dermis of the treated areas and were composed largely of polyhedral-shaped, melanin-containing cells. The authors concluded that the polyhedral cells were probably pigment engorged macrophages. They suggested that dermal melanocytes might also be components of these melanotic foci, but could not distinguish them with the methods employed. Szabó (*19, 46*) observed similar blue nevus-like lesions in pigmented mice after combined treatments with 7,12-dimethylbenz[*a*]anthracene (DMBA) and croton oil. However, he concluded that the dermal melanotic lesions resulted from a displacement of melanocytes from the hair matrices into the surrounding dermis.

Blue nevus-like tumors have also been produced in the skin of golden and white Syrian hamsters after cutaneous applications of DMBA (*56–59*). As little as 50 μg of DMBA in a single application is sufficient to elicit their appearance (*60*). Of many carcinogenic compounds tested thus far, melanotic tumors have only appeared in hamsters in response to treatments with DMBA or urethan (*61*). Ghadially and Barker (*47*) investigated their histogenesis and postulated that they arise from networks of melanocytes situated in the dermis, around pilosebaceous units. Ghadially and Barker suggested that the proximity of dermal melanocytes to the sebaceous glands of hair follicles renders them particularly vulnerable to the action of carcinogens. Studies in our laboratory indicate that the dermal melanocyte networks are associated with the connective tissue sheaths of hair follicles at approximately the level of the sebaceous glands (*48*). The melanocytic networks are amelanotic in white hamsters (*48, 57*). In golden hamsters occasional networks are pigmented, but the majority are nonpigmented (*48*). The nonpigmented perifollicular melanocytes in both white and golden hamsters synthesize melanin in response to repeated cutaneous applications of croton oil (*48*). However, the "activated" melanocytic networks retain their perifollicular arrangement and do not grow progressively to form tumors. This finding suggests that the principal action of croton oil is to increase melanin synthesis while evoking only a limited proliferation of melanocytes. DMBA, in one treatment, is capable of unleashing sustained melanocyte proliferation and, in many cases, a serially propagated capacity for melanogenic activity (*47*).

The histopathology of carcinogen-induced melanotic tumors in hamsters has been described in a number of publications (*47, 58, 59*). The melanotic tumors are highly vascularized, composed mainly of polyhedral- or spindle-shaped cells, and may attain a size of 1.0 cm in greatest diameter. They are clearly dermal in location and invest hair follicles. The tumors appear to begin with a hyperplasia of perifollicular melanocytes followed by their spread both laterally and more deeply into the dermis. The primary association of the melanotic tumors with hair follicles is more difficult to distinguish as the tumors enlarge. Rappaport *et al.* (*59*) have described the morphologic pattern of the tumors as being similar to that of neurofibroma. In the melanotic tumors of golden hamsters, the great amount of melanin pigment often obliterates nuclear and cytoplasmic details. The melanotic tumors of white hamsters appear to be less heavily melanized and range from those with considerable amounts of pigment to those almost entirely amelanotic (*57–59*). Our studies suggest that these tumors may represent mixtures of both melanocytes and connective tissue sheath cells derived from hair follicles (*62*). They are essentially benign lesions but give rise to metastases when transplanted to other hosts (*59*). Ghadially *et al.* (*39*), utilizing a variety of techniques, traumatized the melanotic tumors of both white and golden hamsters, but were unable to convert the benign melanotic lesions into malignant ones.

The histogenesis of spontaneous malignant melanoma in hamsters is strikingly different from that of the chemically induced melanotic tumors. Fortner and Allen (*63*) reported an incidence of about 2 percent of spontaneous malignant melanoma in a large colony of hamsters. In their study of the histopathology of this lesion, they found evidence of junctional nevi in the skin, esophagus, and vagina of normal animals. They concluded that spontaneous malignant melanoma in the hamster arises within such junctional nevi in a manner analogous to the origin of human melanomas. Junctional activity was noted with subsequent invasion of the corium by the tumor cells. Melanotic and amelanotic types of the melanoma developed which, unlike the carcinogen-induced melanotic tumors, regularly gave rise to metastases. However, amelanotic melanomas possessed a greater capacity to grow and to metastasize than the melanotic ones. Fortner and Allen (*63*) emphasize the striking parallel of the growth, microscopic appearance, histogenesis, and biologic behavior of spontaneous melanomas in the hamster and in man. Orkin and Schwartzman (*64*) found certain similarities between human melanoma and spontaneous malignant melanoma in canines.

Collectively, the studies of experimental carcinogenesis in animals illustrate that *primary melanocytic tumors may be derived from melanocytes situated in a variety of niches in skin.* On the basis of his quantitative studies on human melanocytes and experimental studies in animals, Szabó (*19*) suggested that melanomas may frequently arise in human skin from epidermal melanocytes which surround hair follicles and sweat glands. This interesting speculation awaits further documentation.

MELANOCYTES AS SECONDARY FACTORS IN TUMORIGENESIS

Melanocytes may be incorporated as secondary components of such neoplasms as pigmented basal-cell epithelioma, squamous-cell carcinoma, and seborrheic keratoses (*34, 65, 66*). The essential point is that altered epidermal cells form the basic units of structure of these tumors. In some cases most of the pigment is found inside the epidermal cells, with only a small number of weakly dopa-positive melanocytes present in the papillary elongation at the dermoepidermal junction. In others, melanocytes may be found within the tumor mass. The melanoacanthoma (fig. 6) represents a special type of intra-epidermal lesion in which the essential histological character of the tumor depends on the presence of both altered epidermal cells and melanocytes (*66*). In this mixed tumor, melanocytes form integral rather than secondary cellular components. The ratio of altered epidermal cells to altered melanocytes is different in different specimens of melanoacanthoma. The blue nevus-like lesions of the hamster may represent a similar type of mixed tumor. However, further clarification is necessary of the role that connective tissue sheath cells derived from hair follicles may play in their evolution (*62*).

Attempts have been made to relate the gross pigmentation of primary and secondary melanotic tumors to malignancy. It is now generally held that though the amount of melanin varies considerably within human melanomas, the pigment has no influence on the degree of malignancy of melanoma cells (*38, 63*). In contrast, spontaneous amelanotic melanomas in the hamster possess a greater capacity to grow and metastasize than their pigmented counterparts (*63*). Stewart and Bonser (*67*) emphasize the similarity in morphology and clinical behavior of pigmented epithelial tumors to the corresponding types of nonpigmented tumors. Becker (*68*) further stated that melanin appears to have no real significance in human cutaneous carcinoma. Lennox (*69*), however, found an inverse relationship between the pigmentation and growth activity in an extensive series of human squamous papillomas. The most benign tumors contained many melanocytes, the ones most closely approaching malignancy in character, none. Fitzpatrick *et al.* (*70*) observed a direct correlation between tyrosinase activity within melanocytes and their proliferative activity. They suggested that the high level of tyrosinase activity within melanoma cells may indicate biochemical anaplasia.

Similar attempts have been made to relate chemically induced skin carcinogenesis in mice to coat coloration. The literature pertaining to this subject has been reviewed by Little (*71*) and Brenner (*72*). Twort and Twort (*73*) painted mice with carcinogenic oils and tars and obtained evidence that chemically induced tumorigenesis was profoundly influenced by the coat color of the treated mice. Since that time there have been numerous reports of strain differences in chemically induced skin carcinogenesis, where the mice also exhibit characteristic differences in coat coloration (*71, 72*). Gruskay and Strong (*74*) reported that certain recessive genes of mice conferred resistance to the induction of tumors by subcutaneously injected methylcholanthrene. The effect appeared to be additive with increasing resistance paralleling an increasing accumulation of recessive genes. In contrast, Brenner (*72*) concluded that the induction of skin tumors by cutaneously applied methylcholanthrene does not depend on the color of the mice, but rather on constitutional factors not associated with those controlling coat color. Rusch and Baumann (*75*) found a considerably lower incidence of skin tumors in the ears of pigmented mice than in those of albino mice exposed to ultraviolet light. In this case it is conceivable that melanin within the epidermis of pigmented mice served to protect against tumor induction by this agent (*76*). However, possible differences in thickness of the corneal layer or strain differences in constitutive genetic factors might also have accounted for the results observed.

CELLULAR INTERACTIONS DURING CARCINOGENESIS

Pinkus and coworkers (*21*) have emphasized the symbiotic relationship existing between melanocytes and epidermal cells under normal and pathological conditions in man. There is increasing evidence that melanocyte proliferation and pigmentary metabolism depend, at least in part, on cues from epidermal cells. Melanocytes in turn provide epidermal cells with a complement of pigment granules. Burgoyne *et al.* (*45*) have shown that melanocyte hyperplasia and increased melanin synthesis frequently accompany experimentally induced hyperplasia of epidermal cells in mice. An analogous situation in man is the melanocyte hyperplasia and increased melanin synthesis which may accompany the genesis of epidermoid tumors. The melanoacanthoma described by Mishima and Pinkus (*66*) is a particularly striking example of this relationship of melanocyte proliferation to epidermal hyperplasia. Enlarged dendritic melanocytes are distributed throughout this tumor, whereas melanocytes normally are restricted to the junction of the epidermis and dermis. In this sense, the melanoacanthoma is comparable to epidermis treated with thorium X (*21*). In both conditions, the normal barrier against superficial epidermal penetration by mature melanocytes is lowered and—importantly—the release of melanin granules to epidermal cells appears to be blocked (*21, 66*).

It is possible that the origin of primary melanocytic tumors is also dependent on cues from epidermal cells, but no evidence of a definitive nature is available at present. External carcinogenic stimuli including X rays, ultraviolet light, and chemical carcinogens impinge first on epidermal cells before reaching melanocytes. Similarly, intrinsic or systemic factors must strike many types of cells before contacting melanocytes. It is worth considering the possibility that the stimulus for malignant transformation of melanocytes does not reside solely in the carcinogenic agent, but also is related to factors released by other cells. It is clear that the melanoma problem cannot be detached from the general problem of the biology of skin and most particularly that of the epidermis.

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Plate 63

Figure 1.—Human nevus cells (reproduced from Mishima (*36*) with permission of author and publisher). Reduced approximately 30 percent from magnification. × 1,100

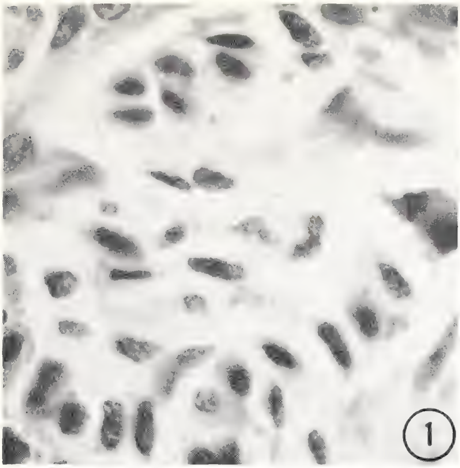
Figure 2.—Melanosis circumscripta praecancerosa (Dubreuilh) with hyperplasia of "clear cells" (melanocytes). *Compare* with nevus cells shown in figure 1 (reproduced from Mishima (*36*) with permission of author and publisher). Reduced approximately 15 percent from magnification. × 913

Figure 3.—Human melanoma cells (reproduced from Mishima (*36*) with permission of author and publisher). Reduced approximately 30 percent from magnification. × 1,100

Figure 4.—Epidermal preparation from malignant melanoma (primary lesion) which arose in human breast. Epidermis has been enzymatically separated from dermis and is viewed from inner surface. *Note* irregular finger-like epidermal downgrowths (reproduced from Szabó (*19*) with permission of author and publisher). × 45

Figure 5.—Epidermal preparation from the normal breast skin adjacent to that of malignant melanoma illustrated in figure 4. *Contrast* normal pattern of breast epidermis with that of malignant melanoma (reproduced from Szabó (*19*) with permission of author and publisher). × 45

Figure 6.—Melanoacanthoma treated with Masson's ammoniated silver nitrate for demonstration of premelanin. *Observe* characteristic hyperplasia of melanocytes and epidermal cells in this lesion (reproduced from Mishima and Pinkus (*66*) with permission of authors and publisher). Reduced approximately 25 percent from magnification. × 135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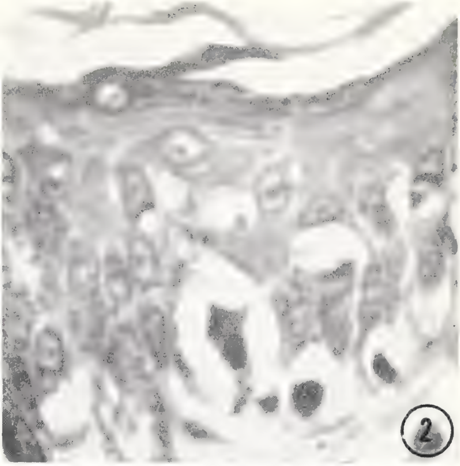
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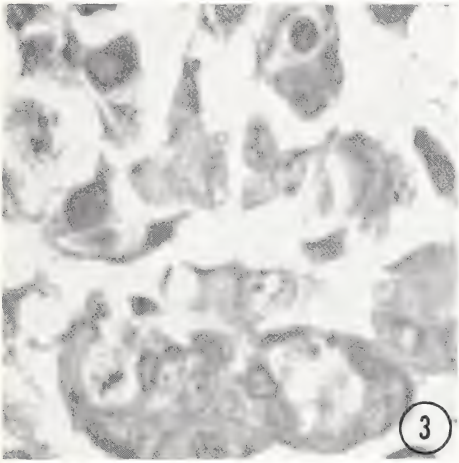
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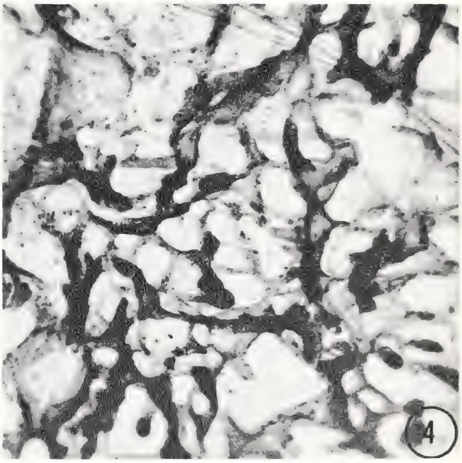
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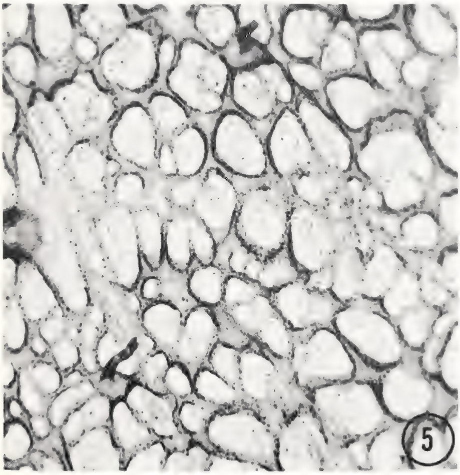
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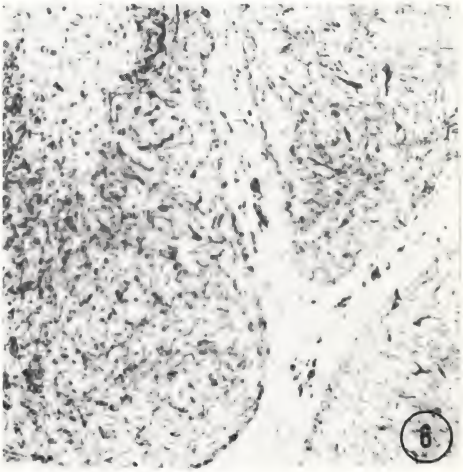
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THE SKIN AS AN ASSAY SYSTEM FOR POTENTIAL CARCINOGENS[1]

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SUMMARY

Even though the skin is the most important, most useful, and most used bioassay system for the determination of carcinogenic properties of physical and chemical agents, it has left unanswered many important questions on cutaneous carcinogenesis. The intriguing problems of species and tissue specificity, such as their relation to the exogenous and endogenous factors determining the causative mechanism active in the process of cellular cancerization and their association to strain and individual susceptibility, have remained almost totally obscure. Available evidence has yielded only fragmentary and often confusing and contradictory information on the factors that seem to determine the carcinogenic properties and potency differences of carcinogenic agents. Bioassay is at present the only reliable method for the determination of carcinogenic properties in members of the rapidly growing mass of natural, semisynthetic, and synthetic chemicals introduced into the human economy and provides thereby valuable warning signs useful in the development and application of preventive and protective measures against potential cancer hazards. Although carcinogenic evidence obtained from bioassays can qualitatively and quantitatively be applied to man only with distinct limitations, it deserves, nevertheless, serious consideration because of the established close agreement between observations made on the effects of occupational carcinogens in man and experimental animals. The value and usefulness of carcinogenic potency ratings would greatly be enhanced by the development of standardized procedures in quantitative screening operations and by the adoption of a uniform evaluation system of generally accepted criteria of carcinogenic potency.—NCI Monogr. No. 10: 577–610, 1963.

BEFORE THE era of modern experimental research in chemical and physical carcinogenesis, the skin of man served as the only available bioassay system for environmental carcinogens. Since observations were not the result of well-planned and controlled experiments, but rather chance findings obtained usually on population groups with a fluctuating and nonstandardized membership that was sometimes exposed to ill-defined agents acting with undetermined intensity, such screening procedures had serious methodologic defects. They yielded data that were essentially only of qualitative nature [Hueper (*1–3*)]. Despite their obvious shortcomings, the etiologic and epidemiologic data derived from them subsequently proved of distinct and fundamental value in the development of more exact, properly standardized, better controlled, and more elaborate bioassay methods in which the skin of experimental animals was used as the test organ for potentially and actually carcinogenic physical and chemical agents.

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SCIENTIFIC AND PRACTICAL SIGNIFICANCE OF CARCINOGENIC BIOASSAYS

No single theory satisfactorily accounts for the carcinogenic activity of all polycyclic aromatic hydrocarbons and their derivatives or applies to all types of known physical and chemical carcinogens [Badger (*4*); Moodie and Reid (*5*); Boyland (*6*); configuration, shape, and size of molecule, Bergmann (*7*); structural symmetry of compound, Lettré (*8*); electron distribution, Pullman and Pullman (*9*); Sayre and Friedlander (*10*); structure and activated phenanthrene-type bond, Haddow (*11*); complex formation with cellular proteins, Miller and Miller (*12*); Heidelberger (*13*); specific chemical reactivity, Fieser *et al.* (*14*); specific metabolite formation, Boyland (*15*); oxidation rate with perbenzoic acid, Eckardt (*16*); photosensitization, Calcutt (*17*); chemoluminescence, Anderson (*18, 19*); cellular fermentation, Warburg (*20*); free radical formation, Syrkin (*21*)]. Slight shifts in the composition and configuration of carcinogenic polycyclic aromatic hydrocarbons are followed by major changes in carcinogenic activity [Dunlap and Warren (*22*); Arcos and Arcos (*23*)]. Similar effects have hydrogenation and oxidation on some, but not all, types of carcinogenic aromatic chemicals [Kotin, Falk, Lijinsky, and Zechmeister (*24*); Lacassagne, *et al.* (*25*)]. Because of the present inability of reliably recognizing carcinogenic chemicals by specific physicochemical properties or biochemical effects [Hackmann (*26*)], bioassays are the only means of obtaining this information.

Carcinogenic bioassays in which the skin or other organs and tissues of experimental animals serve as test systems are the most important and essential procedures for the discovery of carcinogenic agents in the human environment or produced by the human body. Bioassays provide the bulk of the evidence on which protective measures are based for safeguarding the health of the population against cancer hazards from exposure to natural and man-made carcinogens [Hueper (*27–30*); Shubik and Sice (*31*); Food Protection Committee (*32*)]. The development and application of rational and effective preventive measures against occupational cancer hazards in industry are likewise dependent to a large degree on information derived from bioassays [Hueper (*33, 34*); Scott and Williams (*35*); Eckardt (*16*)].

The distinct scientific and practical value of bioassays in such matters has definitely been demonstrated by the close correlations established between the carcinogenic effects produced by occupational carcinogens in man and the identical or similar responses elicited by these agents in experimental animals [Hueper (*29, 33*)]. This conclusion has recently been confirmed again by the demonstration of the carcinogenic action of hydrogenated coal oils (Bergius oils) and of mustard gas on the human skin and respiratory system, respectively—some years after cancers were produced with these chemicals in the skin of rats and mice [Hueper (*36*); Sexton (*37*); Heston (*38*); Yamada *et al.* (*39*)]. There are thus adequate and compelling reasons for one to consider agents capable of producing cancers in experimental animals as potential human carcinogens.

SUITABILITY OF THE SKIN AS A BIOASSAY SYSTEM

The skin offers several distinct advantages over other organs and tissues as a bioassay system for potential carcinogens. These account for its continued predominant use as the test and target organ not only for the screening of chemical and physical agents for carcinogenic properties but also for the investigation of many additional aspects associated with their causative mechanism, their action on special tissue elements, their interaction with specific chemical constituents of the cells and intercellular substances, their metabolites, and their absorption and removal from the site of primary deposition. Because of the accessibility of the skin to direct, accurate, and repeated inspection and the relative ease of its manipulation, this organ system is more suitable and convenient for carcinogenic bioassays than any other.

Test agents can be administered to the skin by the cutaneous, percutaneous, intracutaneous, and subcutaneous routes, and their immediate, chronic and delayed effects can be ascertained by direct visual observation, by physical and chemical procedures directly applied to the skin, and by the removal of tissue exposed to the agent for histologic, chemical, and physical study. The skin, moreover, lends itself to investigations of the influence that carcinogenic and noncarcinogenic agents, introduced into the animal organism by cutaneous and noncutaneous routes, may exert on the cancerization process elicited by carcinogenic agents either directly applied to the skin or introduced by noncutaneous routes. Urethan [Ritchie (*40*)], 9,10-dimethyl-1,2-benzanthracene,[3] methylcholanthrene, 3,4-benzpyrene,[4] 1,2,5,6-dibenzanthracene[5] given intraperitoneally, and 2-acetylaminofluorene[6] administered orally cause a systemic initiating effect of the skin realized by cutaneous applications of croton oil acting as a cocarcinogen [Berenblum (*41*)].

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[3] *Chemical Abstracts'* nomenclature: 7,12-dimethylbenz[*a*]anthracene.

[4] *Chemical Abstracts'* nomenclature: benzo[*a*]pyrene.

[5] *Chemical Abstracts'* nomenclature: dibenz[*a,h*]anthracene.

[6] *Chemical Abstracts'* nomenclature: *N*-2-fluorenylacetamide.

Cutaneous bioassays permit, moreover, investigations on the systemic carcinogenic effects exerted by carcinogens applied to the skin and becoming manifest in remote organs, such as the lungs, hematopoietic tissues, liver, and bladder [Kennaway *et al.* (*42*); Takizawa (*43*); Schabad (*44*); Lacassagne *et al.* (*45*); Andervont (*46, 47*); Andervont and Shimkin (*48*); Andervont and Lorenz (*49*)]. Such cancerous reactions in remote organs and tissues are of special importance whenever the carcinogen tested fails to elicit cutaneous neoplasms at the site of its application. Since cancers usually develop at sites where a particular carcinogen exerts its most intense and prolonged effect, the skin provides a bioassay system that responds to most carcinogenic agents applied directly. There are, however, notable exceptions. In these, the skin demonstrates its unsuitability of providing information on the carcinogenicity of the test material. Some of these agents, such as various carcinogenic aromatic amines, pass so rapidly through the skin that carcinogenically submarginal concentrations in the tissue of primary contact are produced. On the other hand, cancerous responses develop under such conditions at remote sites where the concentration and duration of exposure to these agents are adequate. Other agents, such as metallic nickel or chromium, do not penetrate into the epidermis and, therefore, become biologically unavailable and remain carcinogenically inactive for the skin.

A third variety of carcinogens elicits acute inflammatory and necrotizing reactions in the skin that apparently suppress the development of cancerous cellular proliferations. The chrome holes of the skin elicited by contact with corrosive chromium salts provide an example of this type of carcinogen for which the skin is an unsuitable bioassay system [Hueper and Payne (*50*)]. Whenever the carcinogenic action of a particular test material depends on the formation of a specific carcinogenic metabolite and not on the original substance, the inability of the exposed epidermis of the species used to form such a metabolite would prevent the appearance of cancerous response. It has been proposed that the carcinogenic action of several polycyclic aromatic hydrocarbons, such as 3,4-benzpyrene and 1,2,5,6-dibenzanthracene, is dependent on an oxidation metabolite. It may be possible, therefore, that the resistance of the epidermis of the rat and the susceptibility of its subcutaneous connective tissue to methylcholanthrene may reflect such species-specific metabolic differences. This mechanism is apparently absent in rats when their skin is painted with anthramine since they respond to such treatment with the development of papillomas and carcinomas [Bielschowsky (*51*)].

Numerous observations with subcutaneously injected chemicals have shown that materials totally or almost totally insoluble in the physiologic medium of the subcutaneous tissue, such as several chromium compounds, do not elicit sarcomas at the site of deposition. They may, however, contain a carcinogenic component, which reveals its carcinogenic activity when it is introduced in a more water-soluble form. The action of this principle is well illustrated by the sarcogenic responses elicited in the subcutaneous tissue of rats by several hexavalent chromium compounds and some azo- and triphenylmethane dyes with low or moderate water solubility. Members of the same chemical classes that possess a high water solubility, and therefore are rapidly removed from the sites of administration, fail to produce any tumors in such locations. For substances with such physicochemical qualities, the skin is likewise an unsuitable bioassay system for the demonstration of potential carcinogenic properties, if the only criterion considered is the production of cancers in the skin and unless special precautions are taken for prolonging the action of such agents at the site of administration by incorporating them into vehicles exerting a retarding action on their release and thereby producing a depot effect of the test material.

The skin also may prove an unsuitable bioassay system for carcinogens that are firmly adsorbed to carcinogenically inert matter, such as very small carbon particles, unless proper chemical eluents normally not provided by the skin and its secretions are added to the vehicle, which would make the adsorbed carcinogen available for biologic action [Steiner (*52*)].

OBJECTIVES OF CARCINOGENIC BIOASSAYS

With the skin as the test organ, bioassays may be undertaken for many different objectives. These determine to varying degrees the particular type of methodological approach to be used. The following sections note the various objectives selected for study by bioassay methods and the different methodologic considerations that entered into the performance of the particular tests.

Qualitative Testing

Determination of qualitative carcinogenicity of test materials (qualitative screening test) requires the administration of the largest dose tolerated over prolonged periods and given by single or repeated application to the skin or by injection into the subcutaneous tissue. The principal purpose of this test is to ascertain whether a particular agent will elicit a cancerous reaction under the worst possible conditions of exposure during the lifetime of the animals used. It is essential for obtaining valid information from such a test that the development of a cancerous response is not impaired or inhibited by any toxic action of the test material, especially when given in excessive amounts, or of the vehicle used on the animals and that the vehicle itself does not exert a carcinogenic effect. For incomplete, conditional, or weak carcinogens, it may be necessary to add cocarcinogens or carcinogenic potentiators to the test material for demonstrating their carcinogenic properties and to use young or newborn animals.

Although Berenblum (*53*) recently claimed it would be wrong to institute elaborate tests for borderline carcinogenicity and the aim should be to discover and weed out the potent carcinogens from the large and growing number of suspected environmental chemical agents, there exists, for practical reasons, a real and important need for the application of cutaneous bioassays for the demonstration of weak carcinogens [Kotin (*54*)]. It is quite evident from the incidence rates of different types of cancers and the prevailing epidemiologic scatter pattern of cancers that the majority of human cancers is not attributable to potent carcinogens acting on the human population with great intensity. Obviously, however, an inadvertent introduction of a potent carcinogen into the human economy may lead to the development of a catastrophic epidemic of cancer. Such events have so far been observed in man only under exceptional occupational conditions (bladder cancer among dye workers, lung cancer among radioactive ore miners) and have recently occurred on a national scale for dietary reasons among rainbow trout [Hueper and Payne (*55*)].

Quantitative Testing

Determination of the absolute and relative quantitative potency of a carcinogen, when introduced by cutaneous or subcutaneous routes, requires a distinctly more elaborate and complex set of bioassays than that needed for mere qualitative carcinogenic tests. The following factors deserve consideration [Hieger (*56*); Twort and Twort (*57*); Shimkin (*58*); Druckrey (*59*); Calcutt and Powell (*60*); Shimkin and Wyman (*61*); Shear (*62–64*); Cramer and Stowell (*65–67*)]:

Standardization of methods.—A standardized type of administration of the test material to the skin should be selected. One should use a brush, rod, or dropper and apply measured and controlled amounts to a uniform area or into the skin by percutaneous, intracutaneous, or subcutaneous injection or by subcutaneous implantation.

Since carcinogens are not always equally effective in their carcinogenic action on the epidermis and on the subcutaneous connective tissue, to avoid misleading results bioassays must be performed on both tissues. The epidermis of the rat, for instance, is refractory to methylcholanthrene, while its subcutaneous connective tissue is highly susceptible. The carcinogenic properties of an agent, therefore, do not need to equal its sarcogenic ones [Cramer and Stowell (*68*); Suntzeff, Babcock, and Loeb (*69*)].

For strictly quantitative studies the subcutaneous introduction of the test material is superior to its cutaneous application. Even if special precautions are taken to avoid an undue spread of the applied material [Calcutt and Powell (*60*)], the fact remains that the use of a highly volatile and rapidly evaporating solvent as a vehicle of the carcinogen is associated with marked changes in the concentration of the applied carcinogen. The test material, moreover, may stick to the fur of the animals, may in part be removed by scratching or licking, or may chemically be changed under the influence of light and oxygen. Thus the subcutaneous method is more exact and reliable and more often used in mice and rats [Calcutt and Powell (*60*); Shimkin (*58*); Dunning and Curtis (*102*); Andervont

(*47*); Lacassagne *et al.* (*45*)], although this method also is not entirely free from disadvantages (mechanical trauma, sloughing of skin due to necrosis, loss of injected liquid by back pressure).

Determination of minimal effective dose.—For the establishment of the minimal effective dose, the 50 percent tumor dose and the optimal dose yielding the maximal number of tumors per animal and for the entire group of animals exposed as well as the relative length of the minimal and average or mean latent period or induction time must be determined. For this information the test material must be administered to several series of animals receiving graduated doses [Hieger (*56*); Eckardt (*16*); Wolf (*70*); Hackmann (*71*); Bryan and Shimkin (*72*); Upton (*73*)]. These tests are essential to obtain basic data necessary for the construction of dose-response curves and to calculate tumor yield for the dose per unit area exposed.

Significance of latent period.—Of equal importance concerning the length of latent period and the total tumor yield, apart from the relative dose given, is the rate of application of the carcinogen, *i.e.*, whether it is given in a single dose or in repeated doses at variously spaced intervals. Recent studies of Payne and Hueper (*74*) have shown the minimal latent period is lengthened and the tumor yield is markedly increased when graduated doses of 3,4-benzpyrene are administered subcutaneously to mice at monthly intervals in comparison to the latent period and the tumor yield observed in mice receiving the same total amount of carcinogen by a single injection. It seems that the maximal tumor yield is obtained if a carcinogen is tested by the administration of properly small amounts of a carcinogen given at adequately long intervals so that a prolonged effect of addition and summation can be obtained without, at the same time, production of any considerable cumulative effect. The introduction of excessive amounts of carcinogens as a single large dose or as closely spaced repeated doses apparently results in a certain loss of activity of the carcinogen through lack of total utilization of the amount administered in the cancerization process.

For a study of the roles that the hair cycle [Andreasen (*75*, *76*); Wolbach (*77*); Borum (*78*); Berenblum, Haran-Ghera, and Trainin (*79*); Berenblum (*41*)], the suppression of sebaceous glands [Bock and Mund (*80*, *81*); Brooks, Lalich, and Baumann (*83*); Rehak (*84*); Suntzeff, Croninger, Wynder, Cowdry, and Graham (*85*); Suntzeff, Cowdry, and Croninger (*86*); Klinken-Rasmussen (*87*); Breedis (*88*)], and the hair follicles might play in an induced chemical carcinogenesis of the skin [Rigdon (*89*); Liang and Cowdry (*90*); Rhoads, Smith, Cooper, and Sullivan (*91*)] the use of hairless mice and rats may be advantageous.

Significance of age and sex.—Age and sex of the animals used in bioassays also may influence the results of quantitative bioassays on some carcinogens. According to observations of Leiter and Shear (*92*), male mice were more susceptible to the carcinogenic action of 3,4-benzpyrene than female mice. This correlation was not confirmed for 3,4-benzpyrene by Morton and Mider (*93*). A possible effect of age of the animals used on the outcome of cutaneous bioassays is indicated by the recent findings of Pietra *et al.* (*94*) and of Kelly and O'Gara (*95*), who noted that newborn mice injected subcutaneously with a carcinogen of the polycyclic aromatic hydrocarbon type developed lymphomas early and frequently. A similar connotation have the observations of Smith and Rous (*96*), Smith (*97*), and Graffi and Reissig (*98*) on rapid carcinogenesis made with prenatal and neonatal mouse skin exposed to carcinogens and then implanted into adult mice.

Significance of strains.—Marked differences in the reactivity of different strains, particularly inbred strains of mice and rats to induced skin carcinogenesis, have repeatedly been reported. They are of definite importance in the appraisal of the quantitative carcinogenic effect of test agents on the epidermis and the subcutaneous connective tissue [Gilman (*99*); Andervont (*46, 47*); Bryan and Shimkin (*72*); Hieger (*56*); Andervont and Edgcomb (*100*); Lynch (*101*); Dunning and Curtis (*102*)]. Such differences are especially important in the control of reactions to weak carcinogens. Whenever weak carcinogens are tested, therefore, one must use several strains, particularly if inbred strains are employed. It may be noted that the degree and type of susceptibility of an inbred strain to develop "spontaneous" tumors is unrelated to its reactivity in induced carcinogenicity. Because of the genetic homogeneity of inbred strains, they seem particularly suitable, with some reservations [Armstrong and Bonser (*103*)] for use in quantitative bioassays, while general stock strains, which are more representative of the heterozygous general population and its variations in susceptibility to carcinogens, seem preferable for qualitative tests [Eckardt (*16*)].

Although no definite information exists concerning the reasons underlying these strain and species variations in susceptibility to induced cancer of the skin, recent observations suggest that the epidermis of different strains and species seems to differ in the uptake and retention of hydrocarbons [Bock and Burnham (*104*); Cambel (*105*); Graffi (*106*)] and in the type of metabolites formed [Cason and Fieser (*107*)]. More exact and comprehensive information on such factors would greatly aid in the selection of proper strains and species suitable for bioassays of specific test substances.

The important influence of species-specific factors in the control of the qualitative and quantitative response to cutaneously applied carcinogens is evident from the fact that the skin of rabbits is much less reactive to 3,4-benzpyrene than that of the mouse, though both respond well to coal tar. Cutaneous applications of coal tar, 3,4-benzpyrene, and methylcholanthrene do not elicit skin carcinomas in rhesus monkeys [Hackmann (*26*)], though these animals may develop temporarily cutaneous lesions that exhibit histologic changes characteristic of squamous-cell carcinoma. Their consistent spontaneous regression clearly indicates that histologic malignancy observed in bioassays is not always identical with biologic malignancy. This principle may be applicable in varying degrees to chemically induced cancerous lesions of the skin of most species, including man.

Role of solvents and vehicles.—Solvents and vehicles of the test materials used in bioassays may exert, depending upon their physical, chemical, and toxic properties, a nonspecific modifying, stimulating, inhibiting, or retarding action on the carcinogenic response, provided that such effects are not attributable to any specific cocarcinogenic or anticarcinogenic action of such additives [Rusch, Mueller, and Kline (*108*); Dickens and Weil-Malherbe (*109*, *110*); Dickens (*111*); Shear and Leiter (*112*)]. Peacock and Beck (*113*, *114*) stated that the rate of absorption of the carcinogen as determined by the vehicle or solvent controls the rate and time of cancerous reactions. Strait *et al.* (*115*) noted that the coefficient of lipoid solubility of carcinogenic polycyclic aromatic hydrocarbons in the solvents used correlates closely with the recorded variations in carcinogenic effectiveness. The investigations of Weil-Malherbe (*116*, *117*) and of Weil-Malherbe and Dickens (*118*) on the influence of lipoid and nonlipoid solvents on the rate of elimination and the carcinogenic potency of 3,4-benzpyrene after subcutaneous injection into mice, however, have demonstrated the complexity of this still not well-understood correlation. The possible influence of the degree of unsaturation of the different oils and fats used as vehicles on the carcinogenic response also has been studied [Weil-Malherbe (*116*)].

Since observations of various investigators have shown that sarcomas occasionally develop after a subcutaneous injection of lard, olive oil, tricaprylin, tristearin, and arachis oil [Hieger (*56*)], it may be mentioned that carcinogenic substances may be formed from such aliphatic chemicals if they are subjected to appropriate processing procedures, such as heating [Burrows *et al.* (*119*)] which causes the oxidation and polymerization of fatty acids or the formation of epoxides or peroxides.

There is little doubt that the use of lanolin as a vehicle of benzpyrene, 9,10-dimethyl-1,2-benzanthracene, and methylcholanthrene hinders their carcinogenic effect [Rosicky and Hatchek (*120*); Simpson, Carruthers, and Cramer (*121*); Berenblum and Schoental (*122*); Rusch (*123*)], provided the concentration of the carcinogen is sufficiently low. Since a similar "anticarcinogenic" effect is exerted under similar experimental conditions by mineral oil, the action of lanolin is not specific but is probably a resorption phenomenon [Berenblum and Schoental (*122*)].

Influence of cocarcinogens.—Cocarcinogens inadvertently present in or intentionally added to carcinogens or vehicles, such as tars, petroleum derivatives (oils, detergents), cigarette-smoke condensates, croton oil, sesame oil, oil of sweet orange, ethyl stearate, iodoacetic acid, chloroacetophenone, fatty acids, basic fraction of creosote oil, isopropyl phenylcarbamate, polyoxyethylene sorbitan monostearate (Tween 60), dodecane, dodecylbenzene, phenol, and inflammatory exudate [Berenblum (*124*, *125*); Shubik (*126–131*); Klein (*132*, *133*); Salaman (*134*, *135*); Salaman and Roe (*136*); Salaman and Gwynn (*137*, *138*); Berenblum and Shubik (*139*); Horton and Denman (*140*); Gwynn and Salaman (*141*); Holsti (*142*); Setälä (*143–145*); Boutwell and Bosch (*146*, *147*); Carruthers and Johnstone (*148*); Graffi, Vlamynck, Hoffmann, and Schulz (*149*); Hadfield

(*150*); Riska (*151, 152*); Bischoff, Rupp, Turner, and Bryson (*153*); Gellhorn (*154*); Merenmies (*155*); Saffiotti and Shubik (*156*); Roe (*157–160*); Eckardt (*16*); Menkin (*161*); Walpole (*162*)] exert a definite potentiating and accelerating or precipitating effect on the time of tumor appearance and on the degree of tumor yield obtained in carcinogenic bioassays performed on the skin of mice. These effects are particularly striking if a carcinogen is administered in submarginal doses. The presence of these substances, which are also called promoters of carcinogenesis and originally were considered noncarcinogenic, may influence to a considerable degree the results of potency determinations of carcinogens when performed on mouse skin. The skin of other species (rat, rabbit, guinea pig) when exposed to carcinogenic initiators, however, does not respond to the promoting action of most of these cocarcinogens [Shubik (*128*)]. Klein (*132*) reported that croton oil displays its promoting action also in the development of sarcomas in mice given subcutaneous injections of methylcholanthrene.

The scientific and practical significance of the two-phase mechanism of carcinogenesis, *i.e.*, the qualitative irreversible process of initiation of cancerization by "carcinogens" and its quantitative realization under the influence of promoters, developed from these observations has lately become somewhat blurred by the discovery that practically all of the known cocarcinogens have exhibited also, under proper conditions of exposure, carcinogenic properties, *i.e.*, they have assumed initiating qualities [Selye (*191*); Walpole (*162*); Poel (*163, 164*); Della Porta (*165*); Shubik (*131*); Rush (*123*)]. Similar bivalent claims as to promoting and initiating properties have been advanced in regard to the role that thermic burns may assume in the production of skin cancer. Moreover, most chemicals [Rous (*166*); Friedewald and Rous (*167*); Saffiotti and Shubik (*168*)] are "complete carcinogens," *i.e.*, they exert both initiating and promoting action. A few chemicals, such as urethan, are "incomplete" or "conditional carcinogens," *i.e.*, they are initiators only and require the coaction of a promoter for their carcinogenic realization when they affect the skin. Urethan, however, is a complete carcinogen as far as its blastomatogenic effect on the lung is concerned. In commenting on this situation, Eckardt (*16*) noted that "a carcinogen thus may be academically a weak initiator and a strong promoter or a strong initiator and a weak promoter, or a strong initiator and a strong promoter. This is important from a human environmental exposure viewpoint, where such exposures are multiple in number, but highly erratic in length and degree for individual chemicals."

Solvent action in accelerating or promoting the action of carcinogens adds to the confusion, especially when one considers the fact that dodecyl benzol has a potentiating effect when the dose of the carcinogen is high, but lacks it when it is low [Eckardt (*16*)]. Moreover, urethan, which is a multipotential carcinogen, can act as an initiator and as a promoter of carcinogenesis to the skin of the mouse, though it does not seem to act as a promoter of initiating carcinogenic polcyclic aromatic hydrocarbons or of "background" carcinogens accounting for the occurrence of "spontaneous" cancers.

It should be kept in mind that tumors produced by a combination of carcinogen and cocarcinogen differ from those resulting from repeated applications of a complete carcinogen or combinations of carcinogens [Steiner (*169*); Steiner and Falk (*170*)]. Tumors promoted by cocarcinogens show a diffuse distribution, but relatively few progress to the ultimate cancerous stage and many regress and never appear again. Most tumors initiated and promoted by a carcinogen, on the other hand, progress to a definitely cancerous phase and, if they regress, new tumors frequently reappear at the site of regression [Foulds (*171*)]. These differences in the biologic behavior of tumors developing in response to promoters and initiators are in part related to the fact that promoters enhance cell proliferation (hyperplasia) and differentiation [Setälä (*143*); Walpole (*162*); Berenblum (*125*)]. Initiators, on the other hand, cause a delay in cell maturation and thereby rapidly create an irreversible and specifically modified cellular matrix on which the promoters exert their reversible, less specific, and slowly developing proliferative effect.

The scientifically still unsettled character of cocarcinogens and their undetermined role in the induction of cutaneous cancers in man provide a definite indication for one to include cell promoters in the chemical test systems used in cutaneous bioassays to obtain definite and reliable information on these important aspects of chemically and actinically induced cancers of the skin.

Procarcinogenic factors.—Locally and systemically active, procarcinogenic agents also deserve consideration in cutaneous bioassays, since they may influence, when intentionally or unintentionally present, the outcome of such tests by stimulating cellular proliferation (simple hyperplasia), by facilitating the action of carcinogens on the epidermis or subcutis, or by exerting a local or systemic metabolic effect on the exposed skin favorable to the action of carcinogens. Mechanical and chemical traumas of various types (thermic burns, cauterization, caustic injury, scarification, and chemical inflammation) thus may act as nonspecific carcinogenic accelerators, especially during the neoplastic phase [Rusch (*123*)]. Visible light [Morton, Mider, Luce-Clausen, and Mahoney (*172*)], actinic dermatitis, photosensitizers [Büngeler (*173*)], and chemical and bacterial infectious inflammatory processes [Lacassagne (*174*)] seem to have a similar effect.

Investigations of Tannenbaum (*175, 176*) have demonstrated that animals kept on a fat-rich diet develop cancers of the skin more readily on exposure to carcinogens when they are given in suboptimal doses so the modifying effects of the diet are not masked than animals kept on normal or calorically restricted diets. This supporting effect on chemically induced carcinogenesis of a fat-rich diet may have, in part, a systemic, metabolic background; it must be partly attributable to the fact that the fur and skin of such animals become distinctly oily and, therefore, would favor the resorption of carcinogens applied to the skin, thereby intensifying the degree of carcinogenic exposure and accelerating and accentuating its carcinogenic response.

Experiments with different oils and fats used as vehicles of carcinogens have shown that some favor and others impair the action of these agents on the skin. No conclusive experimental evidence obtained by bioassays is available on whether similar differences in carcinogenic reactivity exist when the variously acting saturated and unsaturated fats and oils are given orally, though this aspect might be of distinct practical importance in the causation and control of chemically induced cancers of the human skin.

Virus and chemical carcinogens.—Observations of Duran-Reynals (*177*) and of Rous and Rogers (*178*) indicate that the inoculation of the skin of experimental animals with nontumorigenic and tumorigenic viruses (vaccinia virus; Shope papilloma virus) stimulates the neoplastic response of such a skin to carcinogenic hydrocarbons or the transformation of viral papillomas into carcinomas, respectively. The use of cutaneous bioassays in the elucidation of the exact interrelations existing between carcinogens, which are as dissimilar as viruses and hydrocarbons in the process of carcinogenesis and cancerization, appears especially pertinent because of the far-reaching claims made recently in regard to the frequency of cutaneous viral manifestations in particular (herpes, infectious warts, etc.). It may be noted in this connection that the development of cancers at the site of inoculations of smallpox vaccine is extremely rare according to published reports. Such observations, in addition to others, do not favor the view that the chemical carcinogens merely act as mediating agents to viruses either representing specific tumorigenic agents ubiquitously present as normal cellular constituents in the tissues in a variety of forms according to the different types of tumors produced, or formed possibly *de novo* in the cell by an interaction between the chemical carcinogen and a mutated nuclear or cytoplasmic gene and then not being a parasite in the usually accepted definition [Berenblum (*53*)].

Presensitization techniques and synergism.—A somewhat similar sensitizing and accelerating mechanism useful in the test of the action of adequate doses of chemical carcinogens on the skin was used by Poel (*179, 180*). These investigators pretreated the skin of mice either with a carcinogenically inactivated methylcholanthrene or with submarginal doses of 3,4-benzpyrene, respectively, before applying the test material some time later. Poel observed a distinct hastening and accentuation of the carcinogenic response to the subsequently administered carcinogen when compared with the reactions seen in controls not previously sensitized.

Experimental evidence indicates that such additive or synergistic potentiating effects on carcinogenesis may occur also between carcinogenic actinic agents (ultraviolet radiation, X radiation, β-radiation) and some chemical carcinogens (tobacco tar, methylcholanthrene, coal tar) when applied successively or simultaneously to the skin of mice. The existence of such interrelations for cutaneous or leukemic cancerigenesis, however, is apparently not a consistent phenomenon but dependent on genetic factors of tumor susceptibility [Mixer and Kirschbaum (*181*); Suntzeff, Cowdry, and Croninger (*182*); Kaplan and Kirschbaum (*183*); Lisco, Ducoff, and Baserga (*184*); Franks and Meek (*185*)]. In fact, some results showed that the administration of two dissimilar carcinogens to the skin or subcutaneous tissue resulted in an antagonistic effect inhibiting carcinogenesis.

Carcinogenic antagonism and anticarcinogens.—There is indeed a great deal of experimental evidence that demonstrates the frequent occurrence of a carcinogenic antagonism between two chemical carcinogens or between a chemical carcinogen and a structurally related noncarcinogen. Lacassagne, Büu-Hoi, Lecocq and Rudali (*45*) have reported that the addition of certain weak carcinogens (chrysene; 1,2,5,6-dibenzofluorene) to methylcholanthrene, or of 1,2,5,6-dibenzacridine to 1,2,5,6-dibenzanthracene, inhibited the carcinogenic effect of the strong carcinogen upon the skin. Berenblum (*244*) similarly reported that the administrations of the carcinogenic mustard gas to the tarred skin of mice inhibited cancer formation.

Similar inhibitory phenomena on cutaneous carcinogenesis were observed when noncarcinogenic agents were chosen that were similar to the carcinogenic chemicals in composition and configuration [Riegel, Wartman, Hill, Reeb, Shubik, and Stanger (*186*); Hill, Stanger, Pizzo, Riegel, Shubik, and Wartman (*187*); Steiner and Falk (*170*); Argus, Sakuntala, and Ray (*188*); DiPaolo, Stanger, and Hill (*189*); Büu-Hoi (*190*); Kotin, Falk, Lijinsky, and Zechmeister (*24*)]. It is generally concluded that this inhibitory effect of structurally similar hydrocarbons is attributable to the fact that the two compounds compete for the same groups of cellular constituents, which causes a chemical phenomenon known as spatial blockade or steric hindrance. The inhibitory mechanism active with these chemical combinations differs from that exerted by thio-compounds on hydrocarbon carcinogenesis, because thio-compounds divert the carcinogenic hydrocarbons from binding sulfhydryl groups of cellular constituents and from inactivating, thereby, enzymes depending in their activity on the availability of free sulfhydryl groups [Crabtree (*192*, *193*); Calcutt (*194*, *195*); Lusky, Braun, and Woodard (*196*)]. A similar mechanism underlies the protective effect of thio-compounds against the sulfhydryl-destroying biologic action of nonionizing and ionizing radiations [Hueper (*197*); Hueper *et al.* (*198*); Elson, Goulden, and Warren (*199*)]. The inhibitory action of a calorically restricted diet on induced cutaneous cancers [Tannenbaum (*176*); Boutwell and Rusch (*200*); Bullough (*201*)] may be related, on the other hand, to a lowering of all vital processes under conditions of semistarvation and on the relative lack of building material necessary for the production of new cells.

These observations are of importance not only for purely scientific reasons to bioassays specifically designed for the study of such aspects in experimental animals, but also for practical reasons.

It is essential that special care be taken that experimental animals used in cutaneous bioassays by the presensitization procedure be free from any previous exposure to carcinogens through transplacental transfer, lacteal ingestion, and carcinogenically contaminated cages and feed, if meaningful results are to be expected [Anderson (*202*)].

Since cancers in man and animals are the results of summation effects of carcinogens [Druckrey and Schmähl (*203*)], the observations of Poel (*179*) and of Cramer and Simpson (*204*) on the influence of presensitization on carcinogenic response may explain the wide fluctuations in the length of the latent period of occupational cancers and the degree of cancerous reactions observed among individuals belonging to population groups apparently sustaining a similar degree of exposure to a chemical carcinogen, because it can justly be assumed that the general and industrially employed population becomes exposed to various environmental carcinogens during their entire lifetime.

CARE OF ANIMALS AND TEST MATERIAL

In addition to these general and specific considerations that enter into the planning of bioassays for carcinogenicity of chemical and physical agents in which the skin serves as the test tissue, special attention must be extended to various important precautions in the care of the animals used in these experiments for the prevention of the occurrence of misleading and unnecessarily complicating reactions. Some of these deserve special consideration in cutaneous bioassays. Under no circumstances for such procedures should one use animals raised in wooden cages impregnated with creosote or previously used in experiments that caused their permanent contamination with carcinogenic material.

The use of separate cages for individual animals offers distinct advantages, since this arrangement prevents loss of animals through injuries caused by fighting among animals and by transfer of bacterial and parasitic infections. It, moreover, eliminates the possibility that such traumas to the treated areas of the skin may act as cocarcinogenic factors. This practice of fighting, frequent among animals, may help to transfer carcinogenic material to untreated lacerated and scarified parts. The use of individual cages reduces also loss of test material from the painted area as well as its spread to other parts of the body and to other animals through licking and through the common clustering together of several animals [Booth and Boutwell (*205*)]. It may be advisable to immobilize animals for a time immediately after they are painted until the solvent has evaporated and the primary irritative reaction has worn off, and if indicated, then the painted area should be covered with a plastic shield [McCarter (*206*, *207*); Calcutt and Powell (*60*)].

It is essential, moreover, that animals used in cutaneous bioassays be as young as possible so that they can be exposed, if necessary, to the test material for a maximal period of time and provide a maximal latent period. Removal of the hair by clipping is preferable to shaving so as to avoid cutaneous injury and infection.

Since animal species frequently develop, like man, so-called "spontaneous" tumors of unknown causation, it is imperative that an adequate number of animals of the same species, strain, age, and sex as those used in the bioassay are included in the experimental procedure. These controls should consist of untreated animals as well as animals given the vehicle or subjected to any physical or chemical factor (heat, light, diet, surgical trauma) to which the test animals are subjected in the bioassay.

For the investigation of certain aspects of cutaneous carcinogenesis by bioassays, the use of congenitally hairless mice or rats may be advantageous, since these possess atrophic hair follicles, sebaceous glands, and a hyperplastic and cornified squamous epithelium.

The rooms should be air conditioned for the best conditions for the animals' long-term survival. Whenever possible animals used in several carcinogenic experiments should not be housed in the same room. Under no circumstances should control animals be kept in the same room with the test animals. The use of pesticides in such rooms and the administration of parasiticides to the test animals are likewise contraindicated since their toxic effects may influence the carcinogenic response to the test material [Eckardt (*16*)]. The feed should be free from carcinogenic, cocarcinogenic, hormonal and toxic ingredients, impurities, and contaminants and should be quantitatively and qualitatively balanced, adequate, and uniform throughout the bioassay. The water and air available to the test and control animals should be as free as possible of carcinogenic or cocarcinogenic pollutants, such as soot and detergents. It seems also desirable to prevent coprophagia, since urine and feces may be contaminated with carcinogenic material resorbed through the skin or from the subcutis. The instruments, glassware, and solutions used for the administration of the test material should be free from any external contaminants. Test material should be prepared at frequent intervals for avoidance of any destruction of the active ingredients by interaction with substances contained in the vehicle or from exposure to air, light, and heat.

These precautions are of special importance whenever weak carcinogens are tested for the reduction of the occurrence of so-called "spontaneous" or "background" tumors [Shubik (*127*)] in both the test and control series, which may not always be of hereditary or congenital origin, but attributable to contact with carcinogens contaminating inadvertently the environment of the experimental animals during, or even before, the performance of the bioassay.

In the storage of specimens representing mixtures of hydrocarbons and other chemicals, such as tars, adsorbates of water pollutants on activated carbons, particulates of air pollutants, oils, fats, commercial feeds, and similar organic chemical mixtures, it is essential to observe special precautions against the occurrence of chemical changes that may definitely influence the outcome of screening tests. Whenever such materials contain chemicals that are light-sensitive, such as 3,4-benzpyrene, they must by stored in bottles of dark-brown glass. If chemical constituents readily react with oxygen of the atmosphere, nitrogen must be introduced into the container and a gas-tight seal provided, such as that furnished by dipping the stoppered bottle into liquefied paraffin. Some materials are not suitable for any prolonged storage, since various chemical constituents may undergo spontaneous chemical changes, especially if they are of the unsaturated type (fatty acids) or have a tendency to polymerize or to interact with other chemical constituents of the mixture. Such materials should be kept in the freezing compartment of a refrigerator until used.

Whenever chemicals are subjected to bioassay procedures that have been obtained by adsorption on activated charcoal or other adsorbents, it is obvious that the eluents released from the adsorbent by treatment with fat or water-soluble solvents represent only a fraction of the chemicals originally present in the crude material passed through the adsorbent as well as of the chemicals adsorbed. The eluted chemicals also may not represent entirely in chemical respects those originally present, since they may have undergone changes brought about by the surface effect of the adsorbent and by the elimination of some chemicals in the mixture. The interpretation of the results of carcinogenic bioassays performed on eluates of adsorbates, therefore, is subject to definite limitations concerning the carcinogenic properties possessed by the crude material from which the eluate was obtained.

BIOLOGIC CRITERIA OF CANCERIZATION OF THE SKIN

Two fundamentally different types of methods have been used in bioassays for the determination of the potential and/or actual cancerization of the skin exposed to chemical carcinogens.

Rapid Tests With the Use of Noncancerous Criteria

In attempts to shorten the observation periods of bioassays, tests have been devised in which various noncancerous-tissue reactions developing within a few days or weeks are used for prognostication of the ultimate malignant or nonmalignant nature of the lesions that might develop from exposures to a particular test material on prolonged contact and survival. Among the various criteria used in carcinogenic appraisal of such rapid bioassays, the following deserve mention: suppression of sebaceous glands in the skin of mice, atypical regenerative regressive epidermal proliferations in the tails of crested newts [Neukomm (*208–209*)], epidermal hyperplasia and acanthosis, increase in number of mitoses, and in size of nuclei and nucleoli, increase in nuclear chromatin and in its deoxyribonucleic acid and ribonucleic acid content [Schaaf (*210*); Cooper and Reller (*211*); Mottram (*212, 213*); Iversen and Edelstein (*214*); Pullinger (*215*); Bock and Mund (*80*)].

It has been claimed by Pullinger (*215*) and others that the initial epidermal hyperplasia of the skin of mice produced by chemical carcinogens differs histologically from that produced by noncarcinogenic hyperplasiogenic irritants, which includes cocarcinogens [Setälä (*143*); Salaman and

Gywnn (*138*); Ritchie, Leroy, and Shubik (*216*)], by exhibiting irregularities in the sizes of nuclei, and distortions of the arrangement of cells, with a mixture of immature cells and completely or partially differentiated cells containing keratin globules. According to Berenblum (*53*), urethan, acting as an initiator of epidermal cancer, does not cause any hyperplastic changes in the treated skin and for this reason epidermal hyperplasia is not essential in the action of a carcinogen. For indication of the apparent diagnostic and prognostic value of such changes, it should be emphasized that atypical hyperplastic and metaplastic epithelial changes in the epidermis and mucosal lining of the upper-alimentary tract, the trachea, the bronchi, and the urinary bladder of man are rather common, and they do not lead, in most instances, to the development of cancerous reactions.

The significance of the shift in the mineral content of the mouse epidermis after the application of chemical carcinogens observed by Suntzeff, Carruthers, and Cowdry (*217*) as evidence of cellular cancerization is not known. A similar judgment is at present indicated concerning the significance of the disturbances in the distribution of -SH and -S-S-groups observed by Setälä (*143*) in the skin of mice treated with 9,10-dimethyl-1,2-benzanthracene when examined with a polarizing microscope. Similar quantitative fluctuations in the sulfhydryl content of the hamster cheek pouch after exposure to 9,10-dimethyl-1,2-benzanthracene were observed during the early hyperplastic stage by Morris *et al.* (*218*). Since none of these histologic and histochemical manifestations is entirely specific for precancerous reactions with obligatory cancerous potentialities, but may follow upon exposure to noncarcinogenic chemicals, the principal value of these quick tests is restricted to their usefulness as screening procedures for the determination of the chemicals most suspect of possessing carcinogenic properties and appropriate for subsequent investigation by the second type of bioassays in which appraisals are based on the demonstration of definite histologic and biologic malignancy.

Long-Term Tests With the Use of Cancerous Criteria

In the ordinary type of cutaneous bioassays conducted for demonstration of the carcinogenic activity of the test material, it is necessary in most instances to apply the suspected agent for the entire lifetime of the animals and to establish by histologic examination the malignant character of the lesions produced, if an agent is to be considered carcinogenic. While the histologic methods and criteria used in such tests are identical with those used in the diagnosis of human tumors, distinct problems may arise at times in the interpretation of some observations as to their significance in carcinogenic respects and may require additional elucidation.

Papillomas as evidence of carcinogenicity.—It may occur that a test agent elicits histologically benign papillomatous growths of the skin. It has remained controversial whether such proliferations should be considered adequate evidence attesting to the carcinogenic character of the agent involved. There should be no doubt as to the decision in such a matter. A papilloma is not a cancer and, therefore, the exclusive production of such benign tumors is not satisfactory proof for the carcinogenic nature of the test material. The appearance of a benign tumor of the papillomatous variety furnishes, on the other hand, proper indication for a restudy of the test material on a larger number of animals and, if necessary, by other experimental methods and under intensified conditions of exposure (cocarcinogen) for the exclusion of the possibility that the test material might nevertheless be carcinogenic.

Since papillomas in induced carcinogenesis sometimes are precursor lesions of cancers, which then usually arise at the base of such benign growths, carcinomas of the skin in man and experimental animals caused by physical and chemical carcinogens arise more frequently *de novo* in other parts of the exposed epidermis. Benign tumors thus do not represent an obligatory stage in the development of cancers [Steiner (*169*)].

Histologic and biologic carcinogenicity.—Epidermal proliferations produced in rabbits and monkeys after they are painted with coal tar have failed to show progressive growth despite the fact that they exhibited cellular and structural changes ordinarily considered as indicative of a carcinomatous lesion. In fact, they regressed, despite a continued application of tar, in monkeys or when tar paintings were discontinued in rabbits, *i.e.*, they were lacking the qualities of biologic malignancy [Foulds (*171*)].

The problem of distinguishing on histologic grounds between chemically induced epidermal lesions possessing biologic malignancy and those displaying merely histologic malignancy recently has acquired practical importance with the increased recognition of self-healing epitheliomas of the skin known as keratoacanthomas that often resemble closely squamous-cell carcinomas. These have been observed in workers after occupational contact with hydrogenated coal oils [Sexton (*37*)] and seem to arise in the superficial parts of the hair follicles of experimental animals after cutaneous applications of tar and carcinogenic hydrocarbons [Ghadially (*242*)]. Since some of these epithelial neoplasms ultimately become malignant, it is again evident that histologic methods and criteria alone may at times be inadequate for the definite establishment of the carcinogenic nature of some particular test material.

Focal or diffuse cancerization.—It has remained controversial whether a single application of a submarginal dose of chemical carcinogens creates a few scattered foci of dormant cancer cells [Berenblum (*53*)] or whether there occurs a more diffuse type of epidermal cancerization. This problem *per se* is of distinct scientific significance. It presents the additional practical question: Do cancers arising under the impact of a subsequently imposed second stimulus from foci of such a sensitized epidermis originate entirely from such dormant cancer cells or do some of the cancerous growths result from a more diffuse cancerous transformation of contiguous sensitized epidermal cells located within the primarily exposed tissue?

Epidermis versus dermis as primary target.—Experimental and histologic studies of Orr (*219*) on mouse skin painted with methylcholanthrene and 9,10-dimethyl-1,2-benzanthracene, involving also the transplantation of the painted epidermis as well as full-thickness skin grafts into nonsensitized parts of the skin, indicated to him that the initial carcinogenic effect of chemical agents is not on the epidermis, but on the connective-tissue elements of the dermis. The changes in the collagen and elastic fibrils and the ensuing fibrosis could be correlated in his opinion with the relative potency of a carcinogen. Epidermal cancerization is, according to Orr (*219*), precipitated by the ischemia of this tissue resulting from the dermal fibrosis. Kreyberg (*220, 221*) has pointed out, in support of this concept, that rats and guinea pigs whose skin is resistant to epidermally applied carcinogens do not develop any vascular reactions in the dermis after administrations of tar in mice.

In refutation of this concept Berenblum (*53*) noted that cancers develop in the marginal portions and not within the fibrotic area of the skin of mice treated with coal tar and carbon-dioxide snow, while Graffi (*222*) commented that Orr's (*219*) views were not supported by the differences in carcinogenic responses following upon the administration of carcinogens in different doses and concentrations as well as by the rarity of sarcomas in the skin receiving epidermal carcinogenic applications. The provocative nature of Orr's concept may stimulate further studies on the relative role of the epidermis and the dermis in chemical carcinogenesis with the use of the method of separation of the two elements recently developed by Ashley (*223*).

Merits of subcutaneous injection method.—Considerable controversy has arisen in recent years as to the scientific and practical significance of sarcomas produced in the subcutaneous tissue of mice and rats either by often repeated subcutaneous injection of the test material or by its introduction into this tissue as a depot, either in the form of an implant without a vehicle (solid or liquid polymerized compounds, metals, oils, fats, cholesterol), or dissolved or dispersed in a lipide or aqueous medium.

For many years the subcutaneous method of administration of carcinogens had generally been accepted and widely been used in qualitative and quantitative bioassays [Shimkin (*58*); Stewart (*243*); Andervont (*47*); Wolf (*70*); Domagk (*224*); Burrows, Hieger, and Kennaway (*119*)]. The validity of these early observations made with this experimental approach was not challenged for many years, especially since various experiments in which different fats, oils, silica, bouillon, and stones were implanted into the subcutaneous tissue of mice and rats did not lead to the development of cancers on the basis of a chronic mechanical or chemical irritation [Burrows, Hieger, and Kennaway (*119*)]. Similar negative results, with the introduction of a variety of inorganic and organic chemicals which includes synthetic aliphatic fats implanted into the subcutaneous tissue of rats and mice, have more recently been reported by Hueper (*225*) and by Hueper and Payne (*228*), thereby confirming the early observations concerning the lack of a carcinogenic effectiveness of nonspecific chronic irritation *per se* or of a foreign body reaction. Berenblum (*241*) commenting on this issue appropriately stated that "the old, lingering belief that 'irritation' is an underlying cause of cancer can now be dismissed, not merely as being out-dated, but in fact as serving only to confuse what is already a very complicated problem." Wolf (*70*) has rightly termed the method of subcutaneous injection of test materials as the "safer method" because it can more easily be standardized.

The doubts as to the significance of the results obtained by this bioassay technique are related to the more recent observation that the occasional and/or frequent development of sarcomas has followed upon the subcutaneous introduction of a number of substances ordinarily considered to be lacking carcinogenic properties [various oils, tricaprylin, paraffin, sugars, sodium chloride solution, hot water, water-soluble carbon polymers, numerous plastics and metals, and mineral matter (glass) [Hueper (*3*); Eckardt (*16*); Oettel (*229*)].

These findings have led some investigators, particularly those with industrial and commercial associations, to consider sarcomas developing in the subcutaneous tissue as nonspecific foreign body reactions and to question the scientific and legal significance of tumor formation obtained in the dermis of mice and rats by the subcutaneous introduction of test materials [Oettel (*229*); Eckardt (*16*); Food Protection Committee (*32*); Hackmann (*26*)].

Thus Oettel (*229*) claimed that the subcutaneous injection and implantation techniques used in rats could lead to misleading interpretations if local sarcoma formation is regarded as proof of carcinogenic qualities of materials tested. A recent report by the Food Protection Committee (*32*) of the National Research Council contains the assertion that the repeated subcutaneous injection of test substances into rats and mice for ascertainment of their carcinogenic activity is of limited value and of dubious interpretation. Eckardt (*16*) contended that the subcutaneous introduction of test materials into animals as a test for their carcinogenic activity has few corollaries in human experience, that the subcutaneous tissue of the rat is highly susceptible to sarcoma formation, and that it is, therefore, unwise to use this species in this type of experiment. He concluded from these arguments that the subcutaneous and intramuscular methods of bioassay are unrealistic from a practical standpoint since they are associated with the production of repeated traumatic and chemical injuries of the target tissue.

The objections raised by these parties in the United States resulted in the continued approval of several food dyes of the triphenylmethane group and other food additives and medicinal agents (carboxymethyl cellulose, iron-dextran complex) that produced, when tested by this method, sarcomas in a considerable percentage of the rats and mice used [Haddow and Horning (*230*)]. This decision has been maintained despite the fact that in Europe it is generally considered that if a substance is carcinogenic by injection then it should not be allowed as a food additive [Boyland (*231*)].

In a critical assessment of the scientific merits of these allegations, occupational accidents with diesel-oil jets and grease guns have resulted in the subcutaneous introduction of potentially carcinogenic diesel oils and petroleum greases. Repeated, frequent, and prolonged contact with cutaneous carcinogens resulting often in chemical injury to the skin is a pattern common in human skin cancers. Moreover, for many years and to an increasing degree during recent times medicinal agents containing potential carcinogens, such as mineral oil serving as vehicles of vaccines and arsenicals and estrogenic preparations, have been administered by subcutaneous injection. There are thus many corollaries in human experience with the experimental practice of introduction of the test materials used in carcinogenic bioassays by the subcutaneous route. This method is for this and other reasons previously mentioned an integral and essential part of any competent and complete bioassay procedure in which the skin is used as the organ of primary contact.

The fallacy of considering sarcoma formation in the subcutaneous tissue of rats and mice after the implantation of plastics and metals and of regarding such sarcomas as expressions of a somewhat nebulous process of physical carcinogenesis [Oppenheimer *et al.* (*232*); Nothdurft (*233*); Hieger (*56*)] has adequately been presented by recent comprehensive experimental observations of Hueper (*225*) and of Hueper and Payne (*50, 226*) on various water-soluble and insoluble carbon and silicon polymers and several metals. A return to the obsolete and well-discredited concept of nonspecific chronic irritation as a cause of cancer and the adoption of the proposal of Nothdurft (*233*), to drop the definitely established concept of specific chemical carcinogens, would indeed be scientific atavism. While some of the experimental observations that are being cited in opposition to the use of the subcutaneous route in bioassays still await a satisfactory explanation and need further investigation, they do not invalidate the usefulness and scientific merits of this bioassay method.

CARCINOGENIC POTENCY RATING

Numerous reliable and competently executed bioassays, many of which used the skin as the biologic test tissue, have established the fact that the degree of the carcinogenic response to a particular carcinogen varies with the dose and that, therefore, such dose-response data are amenable to biometrical methods of evaluation [Bryan and Shimkin (*72*); Payne and Hueper (*74*); Poel and Kammer (*180*); Cramer and Stowell (*67*); Fieser (*14*); Berenblum (*235*); Wolf (*70*)]. Bioassays performed on different chemical carcinogens and with the use of identical experimental test procedures have demonstrated the existence of definite variations in the relative carcinogenic potency of different carcinogens for the epidermis and subcutaneous tissue [Shimkin (*58*); Twort and Lyth (*234*); Twort and Twort (*57*); Berenblum (*235*); Hueper and Payne (*226*)].

Several methods have been proposed to express such differences in carcinogenic potency demonstrated in cutaneous bioassays in exact numerical terms obtained by various mathematical procedures and evaluation of different criteria and sets of biologic data. All systems proposed and used in such appraisals render at best approximate and in part average values possessing a scientific and practical applicability limited by the type of experimental conditions used as reference standards [Goldblatt and Goldblatt (*236*)]. The degree of susceptibility to a carcinogenic agent varies from animal to animal of the same species and of the same strain, which includes inbred strains, even if taken from the same colony and of the same age and sex. Different carcinogens affect in different degrees the various cellular constituents of the skin and the subcutaneous tissue and, when introduced into the skin elicit, in part, also tumors in remote organs representing additional and significant carcinogenic manifestations that enter into calculations of total absolute and relative potency. There exist, moreover, definite and sometimes marked variations in the susceptibility of the skin of different species to the same carcinogen, thereby posing a further complicating factor.

The degree and time of appearance of carcinogenic responses produced by any carcinogenic agent applied to or introduced into the skin depends on a variety of additional factors, such as the absolute dose, the concentration of the carcinogen in the vehicle, the physicochemical properties of the vehicle, the route and method of administration, the number and spacing of applications, and the duration of exposure. Apart from these general biologic and specific technical variables that enter into the establishment of grades of carcinogenic potency, the type and number of the criteria of carcinogenic potency and the method of their quantitative and qualitative evaluation influence decidedly the ultimate potency rating obtained, even when otherwise standardized experimental procedures are observed [Hieger (*56*)]. The ratings have been expressed either in absolute terms related to one or several selected criteria or have been presented in comparative terms, *i.e.*, in reference to potency values obtained in bioassays on some established carcinogen administered in a given concentration and by the same method [Hueper *et al.* (*227*)].

The following list of criteria and the techniques of assessment used by different investigators illustrate aptly the present rather chaotic status of this important aspect of cutaneous bioassays. The least sophisticated methods of expressing potency ratings have used the percentage number of tumor-bearing animals calculated from the total number of animals used in a particular bioassay or from the total number of animals surviving of such a series at the time the first tumor became manifest [Hieger (*56*)]. The minimal length or the average or mean length of the latent period of tumors has served to other investigators as a measure for rating carcinogenic potency. In the Iball index, these two criteria are being combined and correlated as follows:

$$\text{Carcinogenic index } \frac{\text{Percentage of number of tumors}}{\text{Average latent period in days}}$$

Eckardt (*16*) proposed the following formula for expressing carcinogenic potency:

$$\text{Potency number} \frac{10{,}000}{\text{Time elapsed to appearance of tumors in 50 percent of animals}}$$

The time elapsed for 50 percent of animals to develop tumors is calculated from a plot of the data on log probability paper.

A tumor index (papillomas and carcinomas) proposed by Smith (*82*) divided the number of animals with tumors by the number of animals in the test group less any animal that died without a tumor before 90 days from the start of the experiment. In the calculation of the "cancer index" the same method is used except that only cancers are counted. Shimkin (*58*) calculated the carcinogenic index by dividing the percentage of mice with tumors by the average latent period of the whole group in days, multiplied by one hundred. The "relative carcinogenic potency (P_{MC})" is defined by Horton and Denman (*140*) as the concentration of methylcholanthrene that will produce tumors at the same rate as the test material (complex oil) under comparable experimental conditions. The estimation of quantitative rates by this method is indicated and proper, according to Horton and Denman (*140*), if the growth and survival rates of the mice used in such test are satisfactory. The relative potencies of 7,12-dimethylbenz[*a*]anthracene, of 3-methylcholanthrene, and of 3,4-benzpyrene for the skin of C3H mice are approximately 160, 100, and 50, respectively, if P_{MC} is d/n (d is dosage, n is number of applications per week).

Cramer and Stowell (*67*) and Shubik and Ritchie (*237*) advocated that the carcinogenic potency rating should be calculated from the total number of tumors produced in all animals in relation to the dose used, rather than by the percentage of cancerous animals and the time of induction. This procedure was considered to be more meaningful, since a "100 percent incidence" rate is often not attained even by the use of optimal conditions of exposure. The suggestion of Berenblum (*235*), to use the length of the latent period present when 50 percent of the animals show tumors, is defeated by the fact that a 50 percent tumor attack rate under such circumstances is not attainable with many cutaneous carcinogens. In the system of Berenblum (*235*) for grading carcinogenic potency, provision is made of this fact by proposing for carcinogens optimally yielding tumors in less than 50 percent of the animals, substitution of a factor that is obtained by multiplying the time of appearance of the first tumor by two. This factor presumably represents the equivalent of the approximate latent period at the 50 percent level. The grading is then read off from a graph or, alternately, calculated from an appropriate formula. The system allows for 12 grades of potency. The graph is produced by plotting the latent period at a 50 percent level of tumor yield on a logarithmic scale against the 12 grades plotted on an ordinary scale, and a straight line is drawn to join the points of intersection of Grade XII at 4 weeks with Grade I at 200 weeks of latent period.

The method suggested by Iversen and Arley (*238*), based on theoretical considerations of the relationship between quantum transition and tumor production, is at present too complex and hypothetical for routine use; so is the formula proposed by Miescher *et al.* (*239*).

It is obvious from the data given that, until such time that a general agreement is reached for adoption of a more or less arbitrarily arrived at set of standard conditions of bioassays and potency evaluations, the practical value of potency ratings will remain limited. Because of the known species-specificity of carcinogens, it is not possible for one to make any reliable extrapolations from such experimental estimates concerning the prospective potency of any particular carcinogen in man [Hueper (*33*)]. Any attempt to calculate from such data a "safe dose" or a "virtually safe dose" for man requires not only the use of some arbitrary and highly dubious theoretical assumptions as to the level of "safety" desired, but represents also a highly imprudent extrapolation of experimental data [Mantel and Bryan (*240*)].

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SKIN AS A TEST SITE FOR THE BIOASSAY OF CARCINOGENS AND CARCINOGEN PRECURSORS[1,2]

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SUMMARY

I. 3-Methylcholanthrene (MCA) or 7, 12-dimethylbenz[*a*]anthracene (DMBA), absorbed from the alimentary tract, induced keratinizing epidermoid carcinomas of the skin in SWR and C57BL/6 mice. II. Combinations of skin application and gastric intubation, the avenues of exposure now available for epidernal carcinogenesis, provide newer techniques for utilizing the skin as a test site in carcinogenicity bioassays that are potentially shorter in duration and more sensitive than the methods currently employed. III. The need for reclassification of environmental agents causally associated with carcinogenic hazards is reviewed. A classification is suggested which is based on host-agent interactions. Its categories are: A. topical carcinogens; B. carcinogen precursors; C. persistent hormonal supplements, hormone inhibitors, and antimetabolites. Agents that elicit a carcinogenic response are considered according to these categories, while carcinogenicity biossay procedures currently employed, and the potential biossay techniques herein proposed are analyzed for their merits and limitations as test procedures for each category. IV. The fundamental cause of cancer is seen as the proliferation of normal cells in an environment where inhibitory forces, normally exerted on them by contiguous, or homeostatically related cells, are permanently impaired or absent. Removal of inhibitory forces, according to this hypothesis, is due (*a*) to impairment of inhibitory, homeostatically related cells by their interaction with a carcinogen or (*b*) disruption of intercellular contiguity through which the proliferation of normally functioning cells is physiologically controlled. V. The significance of dose-response relationships in carcinogenesis, and of biologic variation in susceptibility to tumor induction are reviewed as critical factors in skin carcinogenesis bioassays. A wide range in susceptibility to skin tumor induction is described for various strains of inbred mice and emphasizes the need for careful choice of test animal in a skin carcinogenesis study.—NCI Monogr. No. 10: 611–631, 1963.

THE OBJECTIVES of this report are to review relationships of carcinogenic dosage and neoplastic response elicited by repeated application of

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benzo[*a*]pyrene (BP) to the skin of mice, to describe the induction of skin tumors by gastric intubation of 7,12-dimethylbenz[*a*]anthracene (DMBA) and 3-methylcholanthrene (MCA), and in the light of the experimental findings to evaluate known techniques and consider newer ones for employing the skin as a test site in carcinogenicity bioassays.

SKIN CARCINOGENESIS DUE TO CUTANEOUS APPLICATIONS OF A CARCINOGEN

I. Dose of Carcinogen, Strain Susceptibility, and Neoplastic Response

The conventional method for inducing skin tumors is by local application of an agent to the shaved skin of a test animal. The technique favored by earlier investigators involved repeated applications of a dissolved carcinogen by painting it on the skin of an experimental animal with a small brush. Greater uniformity in applying the test solution and a quantitative approach to skin carcinogenesis were achieved by substitution of measured drop applications with a calibrated needle pipette for the paintbrush (*1*). The latter approach was used in the following experiment, which illustrates several factors pertinent to skin as a site for carcinogenicity bioassays.

A. *Materials and method.*—Male mice of the SWR, C3HeB, and A/He strains were used. They received drop applications of approximately 0.0075 ml of benzo[*a*]pyrene in concentrations of 0.002 to 1.25 percent (w/v) in toluene, on the shaved, interscapular skin 3 times a week, until they died or a persistent skin tumor developed. The estimated quantities of benzo[*a*]pyrene delivered in this fashion ranged from 0.15 to 470 μg per application.

B. *Results.*—Table 1 shows the incidence of skin tumors and time of tumor appearance in relation to dosage. For each strain, a threshold dose was apparent below which no tumors were induced despite lifelong repeated exposures. Threshold doses for tumor development are thus demonstrable, as determined by the biologic background of the exposed animal, and not merely by an absolute amount of carcinogen.

Above the threshold dose level for tumor genesis is a range in which premalignant tumors were induced. The dose range for premalignant tumor development depended on the biologic background of the exposed animal: In the SWR strain, the upper limit was between 0.38 and 0.75 μg BP applied repeatedly, for the C3HeB between 0.75 and 3.8 μg, while for the A/He the threshold dose for malignancy was in excess of 3.8 μg.

Above the upper dose level characterized principally by premalignant tumors was a range in which malignant tumors were induced. An increased incidence of epidermal carcinomas was elicited after a shorter period of exposure, was obtained as the dose for repeated exposure was augmented (3.8 μg or more of BP, table 1).

It is thus apparent that the threshold level for the induction of malignant tumors, the threshold range for premalignant tumor development, and the threshold for any tumor induction are dependent in part upon the biologic background of the exposed animal. In each instance the SWR was consistently a more sensitive test animal for skin tumor genesis than the C3HeB, and the skin of the latter was more sensitive to a topical carcinogen than the skin of the A/He.

In the range of high intensity exposure (19–470 μg) virtually all exposed animals developed malignancies. In this range, where no further increment in tumor incidence was possible, a larger carcinogenic dose was reflected in a shortened time for tumor appearance (table 1) and in a marked reduction in the lifespan of the exposed animals (*2, 3*). At the highest dose level of exposure, involving repeated applications of 470 μg, differences in reactivity among the 3 strains were minimal. The most sensitive mice of each strain developed tumors within equivalent periods. The animals that did not develop tumors were those dying prematurely.

C. *Implications of observations.*—These data indicate in part the wide range in susceptibility to skin tumor induction among the various inbred strains of mice now available commercially. Careful choice of the most susceptible and hardy strain of test animal is one obvious way of improving the efficiency of a bioassay based on skin carcinogenesis.

Text-figure 1 indicates a few stages of tumor genesis and spread which are suggested by the data. If α–ω (alpha–omega) represents the process of tumor genesis, the segment bracketed by α-A represents the normal range of tissue reactivity. In it, cellular polarity, hyperplasia, and hypertrophy are coordinated with the physiologic needs of the host, as seen in the epithelium of shaved, toluene-treated controls. A-B represents the stage of premalignant tumor genesis or preneoplasia, in which a tissue summates the biologic effects of carcinogenic stimuli, with no obvious signs of a neoplasm (*2*, *4*). With adequate exposures, this stage continues into the stage of carcinogenesis, B-C, in which premalignant tumors may appear (table 1, tumors indicated by ‡). Premalignant tumors may develop and regress or they may persist, with limited growth potentials in dynamic equilibrium with the host. Death at this stage or earlier is not necessarily related to the presence of the induced tumors. In fact, mice with skin tumors induced at these threshold levels invariably have lifespans exceeding the median for the nontumorous mice of their group or for the controls. Excessive exposure during this stage, or during an earlier stage, leads to the completion of carcinogenesis and to tumor growth that is not coordinated with the physiologic and anatomic needs of the host. The result in this experiment was keratinizing epidermal carcinoma, a growth that parasitizes the host by direct infiltration locally, and in a more advanced stage by spread through metastasis to the regional lymph nodes and lungs. The advanced, malignant growth of this stage apparently has a causal association with the death of the host. At present, the conservative diagnosis of a cancer, or the designation of a carcinogen depends on the induction of a tumor whose growth and spread

TABLE 1.—Incidence of skin tumors and time of tumor appearance, in relation to dosage of benzo[*a*]pyrene*

µg BP applied 3 times weekly	SWR ♂		C3HeB ♂		A/He ♂	
	Number with tumors / Number exposed 10 or more weeks	T.A.T.† range / Median T.A.T.	Number with tumors / Number exposed 10 or more weeks	T.A.T.† range / Median T.A.T.	Number with tumors / Number exposed 10 or more weeks	T.A.T.† range / Median T.A.T.
0. 00	0/20		0/17		0/17	
0. 15	0/25		0/19		0/18	
0. 38	2/22‡	55–55‡ / 55	3/17‡	81–93‡ / 81	0/19	
0. 75	15/18	25–72 / 49	4/17‡	51–93‡ / 84	0/17	
3. 8	12/17	25–51 / 38	11/18	35–73 / 65	0/17	
19. 0	16/16	12–28 / 22	17/17	13–32 / 22	21/23	21–40 / 28
94. 0	16/17	9–17 / 11	18/18	10–22 / 19	11/16	14–31 / 28
470. 0	14/14	5–11 / 10	17/17	4–19 / 9	17/17	4–21 / 10

*Carcinogen applied thrice weekly to shaved, interscapular skin of male SWR, C3HeB, and A/He mice during lifetime on experiment. Mice were 10 to 14 weeks old on initial exposure.

†T.A.T. = Time of tumor appearance, in weeks, after first exposure.

‡Skin tumors were "benign" on histologic examination, or regressed during life of animal.

Controls — No carcinogenic treatment A	Animals exposed to subthreshold doses of carcinogen B	Carcinogenesis C	Propagation *in vivo* of malignancy
Normal range of tissue reactivity	Premalignant tumor genesis or preneoplasia Tissue summation of carcinogenic stimuli with no gross signs of neoplasia	Premalignant tumors develop, and regress, or persist with limited growth potentials, in dynamic equilibrium with the host	Tumor growth and spread not coordinated with needs of the host Growth characterized by its parasitism of host ω
α Cellular polarity, hyperplasia, and hypertrophy coordinated with physiology of host	Median lifespan of treated animals equals or exceeds that of controls	Death not necessarily related to tumor	Death causally associated with malignancy Lifespan is shortened

TEXT-FIGURE 1.—Demonstrable stages of tumor genesis and spread.

are of the latter type (C–ω). Current comprehension of the neoplastic process appears to justify an earlier suggestion *(5, 6)* that the induction of premalignant tumors indicates a weak carcinogen, a potent carcinogen in high dilution, or a carcinogenic mixture of which some components are locally necrotizing.

Minimum time periods within which skin tumors can be induced are indicated in table 1 for the various dosages of BP employed. For the most sensitive strain, SWR, exposed repeatedly to the highest dose, 470 μg, the median exposure period for a tumor incidence of 100 percent was 10 weeks, with a range of 5 to 11 weeks. The high frequency of exposure and large dose of BP which elicited these responses suggest that these values are optimal for brevity of exposure and tumor incidence, in bioassays where the primary objective is the induction of a malignant skin tumor.

II. Enhancement of Weak Carcinogenicity by Pretreatment of Skin With a Potent Carcinogen

When a threshold dose of BP was employed, the exposure period to induce a premalignant tumor was a large fraction of the animal's total lifespan: With threshold levels of chronic exposure, most mice never developed a grossly visible tumor even though they were exposed throughout their entire life and lived to ripe old ages (table 1). However, when a high dose of carcinogen was applied for relatively short periods, an advanced stage of skin sensitization or premalignant tumor induction was achieved within a small fraction of the animal's lifespan *(4)*. Subsequent applications of a relatively weak carcinogen then elicited a heightened neoplastic response, because of the skin's capacity to summate the biologic effects of sequential exposures to carcinogens. The summation of sequential exposures to a strong and weak carcinogen may be illustrated with the data of the following experiment.

A. *Materials and method.*—SWR male mice were "pretreated" on the shaved, interscapular skin with 94 μg of BP, 3 times a week, for 4 weeks. Control groups were pretreated with toluene only. After a rest period of 3 weeks, individual groups of approximately 9 control and 9 BP-pretreated animals were given secondary exposures: These consisted of toluene, 0.01 percent BP in toluene, or 15 percent of a light creosote oil in toluene applied 3 times a week until tumors developed.

B. *Results.*—As seen in table 2, carcinogen pretreatment resulted in a shorter secondary exposure period preceding the first tumor (cols. a), a shorter time for maximum tumor incidence in the group as a whole (cols. b, denominators), and a higher tumor incidence (cols. b, numerators).

C. *Implications of observations.*—Text-figure 1 helps us to visualize the potential advantage of a 2-step carcinogenesis bioassay. In the first stage of exposure, a high dose level of a potent carcinogen acting for a limited period may carry the neoplastic process at a rapid rate to a stage of preneoplasia (A–B) or even to the induction of premalignant tumors of the skin (B-C). In the second stage, the transition from preneoplasia or premalignant growth to malignancy is carried on by the agent undergoing malignant growth to malignancy is carried on by the agent undergoing bioassay, if it possesses carcinogenic activity. It is through the first step, the reduction in time required to reach preneoplasia or premalignant tumor genesis with a standardized potent carcinogen, that economy in time and increased sensitivity of response may be obtained when testing an agent applied in the second step.

TABLE 2.—Enhancement of skin tumor incidence and induction time, by pretreating SWR males with benzo[*a*]pyrene

Pretreatment 1.25% BP in toluene, 94 μg 3 times weekly (wks)	Rest period (wks)	Secondary treatment 3 times weekly					
		Toluene		0.01% BP in toluene		15% Creosote oil in toluene	
		a*	b†	a*	b†	a*	b†
0	3	—	$\frac{0}{112}$	36	$\frac{22}{55}$	38	$\frac{75}{56}$
4	3	25	$\frac{33}{63}$	18	$\frac{62}{56}$	6	$\frac{100}{40}$

*Week of secondary treatment preceding first tumor on most susceptible mouse of group (approx 9 mice/group).

†$\frac{\text{Maximum tumor incidence (\%)}}{\text{Weeks of secondary treatment}}$.

Thus a more sensitive bioassay to detect skin carcinogenicity may be possible by calibrated, local applications of a standardized, potent carcinogen, followed by localized skin applications of the test material.

SKIN CARCINOGENESIS DUE TO GASTRIC INTUBATION OF CARCINOGEN

A second route of exposure for sensitizing the skin to carcinogens was suggested by our recent observation that skin tumors could be induced by absorption of a carcinogen from the alimentary tract. The two brief experiments now reported review the observations of this phenomenon.

I. Skin Carcinogenesis by Absorption of MCA From the Alimentary Tract (7)

A. *Materials and method.*—In the first experiment, 2 groups of SWR female mice received gastric intubations of 0.1 ml of MCA, at a concentration of 1 or 0.25 percent in Tween 60.[4] Two other groups received gastric intubations of BP at a concentration of 1 or 0.25 percent. Control mice received comparable treatments with Tween 60 or physiologic saline. Intubations were made twice a week for the duration of the animal's life. Mice dying during the first 10 weeks of treatment were not included in this report.

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[4] Generously supplied by the Atlas Powder Co., Wilmington, Del.

B. *Results.*—Table 3 indicates that carcinomas of the skin were induced in approximately 50 percent (17/33) of the animals intubated with MCA. In contrast, none of the mice intubated in equivalent fashion with BP, Tween 60, or saline developed any skin tumors.

Characteristic features of several induced tumors are seen in figures 1 through 5. These are keratinizing squamous-cell and basosquamous-cell carcinomas. Their induction could have been predicted if a mouse had received repeated applications of a polycyclic carcinogen on the shaved, interscapular skin. Since it is now apparent that carcinomas of the skin may also be caused by absorption of a carcinogen from the gastrointestinal tract, the extrapolation that a keratinizing epidermal carcinoma *per se* is indicative of direct contact with an external carcinogen is no longer tenable.

Table 3.—Skin carcinogenesis in SWR ♀ mice by gastric intubation of 3-methylcholanthrene dissolved in Tween 60

	Number of mice with skin tumors	T.A.T.* range
Agent intubated 2 times weekly, 0.1 ml per dose	Number of mice intubated >10 weeks	Median (wks)
1.0% MCA in Tween 60	10/17	14–23 16
0.25% MCA in Tween 60	7/16	25–34 31
1.0% BP in Tween 60	0/15	—
0.25% BP in Tween 60	0/15	—
Control, Tween 60	0/16	—
Control, saline	0/16	—

*T.A.T. = weeks of exposure preceding tumor appearance.

II. Skin Carcinogenesis by Absorption of DMBA From the Alimentary Tract

The induction of skin tumors by forced feeding of MCA, but not BP, prompted a second exploratory study, to ascertain whether similar results were possible with another carcinogen and strain of mouse.

A. *Materials and method.*—In the second study the carcinogen DMBA and the mouse strain C57BL/6 were used. Mice of both sexes received individual doses of approximately 3 mg DMBA in Tween 60 by gastric intubation, twice weekly, for 1, 2, or more weeks. Control animals received equivalent intubations of BP, the solvent Tween 60, or physiologic saline.

B. *Results.*—The number of experimental groups was curtailed markedly due to the high mortality among all mice intubated 3 or more times with DMBA. Figure 6 shows a mouse that died with severe generalized edema and ascites less than 1 week after the fourth DMBA intubation.

Because of the high mortality, table 4 presents data on tumor genesis for animals intubated twice only. Of 32 animals given DMBA, 2 died before the first tumor appeared; 10 of the 30 survivors developed skin tumors; none appeared grossly malignant by the 40th week of observation. As in the preceding experiment, no skin tumors were induced by intubation of 2 percent BP, Tween 60, or saline.

Table 4.—Skin tumor induction in C57BL/6 mice by gastric intubation of 7,12-dimethylbenz[*a*]anthracene (DMBA) dissolved in Tween 60

			Skin tumors by 30th week of experiment			
Agent given twice only for 1 week; 0.15 ml	Initial No. of mice		Number with skin tumors / Number exposed 8 weeks or longer		T.A.T.* (wks)	
	♂	♀	♂	♀	♂	♀
2% DMBA in Tween 60	16	16	4/14	6/16	8, 9 20, 20	8, 9, 13 30, 30, 30
					(None malignant by 40th week)	
2% BP in Tween 60	16	16	0/16	0/15	—	—
Tween 60	15	16	0/15	0/16	—	—
Saline	7	5	0/7	0/5		

*T.A.T. = tumor appearance time, after intubation.

III. Implications of Findings

From these data, it is apparent that DMBA and MCA are topical carcinogens that withstand metabolic deactivation after systemic absorption from the alimentary tract. With each agent, multicentric tumors and multiple primary tumors were obtained peripheral to the alimentary tract (*7*, *8*). Specifically with reference to the skin, these experiments demonstrate that DMBA and MCA can induce skin tumors after systemic absorption.

Tables 3 and 4 suggest also that a potent agent like BP may be partially or completely deactivated as a carcinogen after systemic absorption and metabolic interaction with the host. The topical effects of BP after intubation, but prior to its systemic degradation, are demonstrable however by forestomach carcinogenesis. A classic study of the morbid anatomy, histopathology, and histopathogenesis of these tumors was published by Stewart and Lorenz (*9*). Examples of BP-induced carcinomas of the forestomach are shown in figures 7 and 8. Dose response relations for their induction in the mouse parallel those for skin carcinogenesis due to cutaneous application of the same agent (*2*, *10*). The dose levels required for forestomach tumors are greater however than are needed for the induction of comparable skin tumors by cutaneous application of BP (*7*).

DISCUSSION

A. Classification of Agents Associated With Carcinogenic Activity (Table 5)

With the exception of substances described in the literature as "cocarcinogens" or "promoters,"[5] all other exogenous agents associated with tumor genesis to date have been called "*carcinogens*," and classified according to their natural characteristics, *i.e.*, as physical, chemical, or biologic. This classification has been useful only in a limited sense, for the terms carcinogen and carcinogenicity do not characterize an agent, but portray, rather, a biologic reaction elicited by the agent. Let us, for example, consider 3 agents usually classified as "chemical carcinogens": DMBA, β-naphthylamine, and urethan. All 3 are termed "carcinogens," all are chemicals associated with tumor induction, but only DMBA has elicited malignancies in the various species of mammals tested to date. β-Naphthylamine and urethan depend on specific metabolic processes found in some species, but not in others, for conversion to carcinogenic derivatives; some agents like urethan depend also on strain-specific metabolic variables for their conversion. Thus, β-naphthylamine is topically inactive, but its metabolites excreted by man and dog are carcinogenic, while those excreted by the rat are apparently not potent. Similarly, urethan is topically inactive: Administered systemically to some strains of mice it elicits neoplasms; it fails to do so in other strains even after lifelong administration. The designation of these agents as "carcinogens" may therefore be questioned: Equally inadequate or inaccurate is their designation as "distant, or incomplete carcinogens." These apparent inadequacies may be circumvented by a classification of them as *carcinogen precursors*, agents whose metabolic conversion to a potent state are wholly and peculiarly dependent upon the metabolism of the host (B, in table 5).

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[5] Usually a crude mixture containing a carcinogenic component, and a locally necrotizing fraction, *i.e.*, croton oil. Some excellent solvents for polycyclic carcinogens have also been reported to be cocarcinogens: Possible reasons for this error have been discussed previously (*2*).

As regards topical carcinogens (table 5: A-I, II), the differences between the host's systemic interaction with MCA or DMBA on the one hand, and BP on the other, suggest 2 subclasses, whose differentiation depends on whether an agent, like DMBA, is resistant to metabolic deactivation, or whether (like BP) it is susceptible to metabolic degradation after absorption and metabolic alteration.

A third class may be considered (table 5-C): Hormonal supplements, hormone inhibitors, and antimetabolites, agents that induce persistent proliferation of normal target cells either by direct stimulation of their proliferative kinetics, by blockading their related inhibitory cells, or as antimetabolites by creating a persistent biochemical state which induces hormonal imbalance and unchecked hyperplasia. Hormonal supplements may be exemplified by multiple pituitary grafts (*11*) or exogenous estrogen; inhibitors, by thiourea; and antimetabolites, by ethionine. Whether urethan also induces neoplasms as an antimetabolite in competition with urea is yet to be ascertained. Hormone inhibitors like thiourea may, in addition, be considered special types of topical carcinogen, insofar as their effects are mediated by interaction with, and physiologic impairment of cells homeostatically related to those that proliferate and become neoplastic because of decreased inhibition.

When viewed in terms of the classes of carcinogens enumerated, it is apparent that a bioassay technique, or an experimental animal adequate for detection of agents of one category may be wholly ineffective for

Table 5.—Classification of exogenous agents associated with carcinogenic activity

Classification	Definition	Examples
A. Topical carcinogens	Agents which induce neoplastic proliferation of normal cells indirectly, by interaction with and permanent physiologic impairment of contiguous or homeostatically related cells, or by permanent disruption of intercellular contiguity	Ionizing radiation, plastic or metal film; viral and metazoan oncogenic parasites; polycyclic carcinogens
I. Metabolism-resistant carcinogens	Agents that elicit tumors locally by topical application, and resist metabolic inactivation after ingestion and systemic absorption	MCA, DMBA
II. Degradation-prone carcinogens	Agents that elicit tumors locally by topical application, but are degraded in potency after absorption and metabolic alteration	BP
B. Carcinogen precursors	Agents that give rise to topical carcinogens *in vivo*, after metabolic alteration in the body of the host	*o*-AAT, DAB, 4-aminodiphenyl, β-NA, urethan, AAF, and 2,7-diacetylaminofluorine
C. Hormonal supplements, hormone inhibitors, and antimetabolites	Agents that induce persistent proliferation of normal target cells either by direct stimulation of their proliferative kinetics, by blockading their related inhibitory cells, or as antimetabolites by creating a persistent biochemical state which induces hormonal imbalance and unchecked hyperplasia	Estrogenic compounds, thiourea, urethan(?), ethionine(?)

another. A bioassay technique should therefore be evaluated in relation to each class of agent currently recognized. Tables 6, 7, and 8 outline advantages and limitations currently visualized for single-stage carcinogenicity bioassays, and for the proposed techniques which involve 2 stages of sequential exposures and have not yet been validated experimentally.

Table 6 sketches the qualitative and quantitative approaches now available for the induction of skin tumors on mice by exposures to a single agent. Among the limitations of the topical skin approach, in a single-stage bioassay, are its time-consuming, prolonged, and inconclusive course, especially with weak topical carcinogens, and its inadequacy for the detection of carcinogen precursors. Skin tumor induction by the administration of a test substance to the animal via the alimentary tract is a new approach, with potentials that are yet to be explored by the experimental investigator. Otherwise, gastric intubation is an old and practical technique in toxicology laboratories. It is useful for the detection of carcinogen precursors, provided the test animal metabolizes an agent as the human does. It is also useful for the detection of topical carcinogens, although the skin of the mouse is more sensitive to topical

Table 6.—Skin tumor induction in mice: single-stage exposures

Technique	Advantages	Disadvantages
A	Pro	Con
Repeated skin applications of test substance in solution 1. Qualitative—brush application of highest concentration that is not locally necrotizing 2. Quantitative—drop application of known volume and concentration	Simplicity of technique Tumor genesis attributable to 1 agent only Technique is practical and currently in use for detecting topical carcinogens	Time-consuming, and inconclusive with weak agents Not applicable for detection of carcinogen precursors
B		
Repeated gastric administration of test substance 1. Qualitative—*ad libitum* ingestion of known concentration in food or drinking water 2. Quantitative—gastric intubation of measured amounts	A new route for skin carcinogenesis with potentials yet to be explored Useful for detection of carcinogen precursors Differentiates metabolism-resistant agents from those susceptible to metabolic degradation and inactivation An established method for acute and chronic toxicity studies	Systemic toxicity may be a limiting factor of chronic administration Tumor induction in other tissues may precede and inhibit skin tumor genesis Skin tumor induction may be rare, and demonstrable only with agents of high potency Risk of trauma and death of test animal due to intubation technique is greater than that for cutaneous exposure

carcinogens than the forestomach (*7,8*). Oral administration of a test material to detect topical carcinogenicity has not been practiced, though carcinogenesis by feeding MCA has been established as a technique for inducing mammary carcinoma, lymphatic and myelogenous leukemias, ovarian and pulmonary tumors (*12–17*).

Table 7 outlines 4 simple combinations for studying potential techniques of carcinogenesis bioassays involving 2 stages of sequential exposures: a stage of priming or sensitization to carcinogens, and a subsequent stage for administering the test material. Combinations I and III are directly pertinent to the skin as a site for the bioassay of topical carcinogens II and IV appear promising for the detection of carcinogen precursors, hormone supplements or inhibitors, and antimetabolites. Although the potential applicabilities and limitations of each are outlined, (table 8), the value of all 2-stage combinations for carcinogen bioassay remains yet to be determined experimentally.

Table 7.—Four combinations for studying 2-stage carcinogenesis bioassays

		I	II	III	IV
Priming exposure	via	Skin application	Skin application	Gastric intubation	Gastric intubation
Test exposure	via	Skin application	Gastric intubation	Skin application	Gastric intubation

TABLE 8.—Potential techniques for carcinogenicity bioassays: 2-stage sequential exposures

	Currently visualized:	
Proposed technique	Advantages	Disadvantages
I Pretreatment Cutaneous applications of standard carcinogen Test exposure Cutaneous applications of test solution	Accelerated bioassay for topical carcinogens Greater sensitivity for detecting weak carcinogens	Requires pretreatment with high potency carcinogen and a rest period of 2 to 3 weeks before applying test agent Requires more controls and finer technique to differentiate tumorigenic effects of priming and test exposures Not applicable for carcinogen procursors and class C agents (table 5)
II Pretreatment Cutaneous applications of standard carcinogen Test exposure Gastric intubation of test solution	Accelerated detection of carcinogens resistant to metabolic deactivation Detection of topical carcinogens susceptible to metabolic deactivation (by forestomach carcinogenesis but no increase in skin tumor genesis) Detection of carcinogen precursors by systemic effects and possibly by induction of skin tumor No need for rest period between pretreatment (P) and test exposure (T.E.)	Requires pretreatment with high potency carcinogen Requires more controls and finer technique to delineate effect of P. and T.E. Systemic toxicity of agent may limit chronic intubation Tumor induction in other sites may precede and inhibit skin tumor genesis Recurrent risk of trauma and death of experimental animal due to exposure technique Possibly no summation between P. of skin and alimentary tract T.E.
III Pretreatment Gastric intubation of carcinogen resistant to metabolic deactivation Test exposure Cutaneous applications of test material	Unexplored area of carcinogenesis investigation Applicable for study and/or detection of topical carcinogens	Topical application of class C agents or carcinogen precursors is less effective than systemic or gastrointestinal administration
IV Pretreatment Gastric intubation of carcinogen resistant to metabolic deactivation Test exposure Gastric intubation of test agent	Unexplored area for cancer research May contribute to an understanding of non-neoplastic pathology, and pathology of tumors currently considered cryptogenic; *i.e.*, generalized anasarca, edema, ascites, retroperitoneal tumors, etc.	Probability of developing nonepidermal systemic malignancies in or peripheral to the gastrointestinal tract is greater than that of inducing skin tumor Systemic effects of the pretreatment or test exposure may inhibit the neoplastic effects of the other, *e.g.*, MCA inhibition of AAF or di-AAF hepatomagenesis

Unusual, non-neoplastic lesions have been seen among some experimental animals given the highest dosage of carcinogen by gastric intubation: Their deaths were associated with immobilization of the peritoneal organs by adhesions, probably secondary to a nonsuppurative chemically induced visceral peritonitis, and mesenteritis (fig. 9). The etiology is easily visualized by exposing the peritoneal contents of an intubated mouse to ultraviolet illumination and noting the fluorescence of the mesenteric fat and mesenteric lymphatics within 1 or 2 hours after forced feeding of the polycyclic carcinogen. Non-neoplastic deaths associated with generalized anasarca and ascites have already been mentioned (fig. 6). Survivors have developed rare tumors whose etiologies were previously obscure: dermal sarcomas, sarcomas of the mesentery, and sebaceous-gland adenomas and carcinomas (figs. 10 through 16). These observations, together with the potential development of more rapid and sensitive bioassay techniques, indicate that further investigation of the alimentary tract as a route for carcinogenic exposure may be of interest to physiologists, toxicologists, and investigators of non-neoplastic pathologies, as well as to the experimental carcinogeneticist.

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PLATE 64

FIGURE 1.—Keratinizing epidermal carcinomas on virgin SWR female mice treated by gastric intubation of 1 mg MCA in 0.1 ml Tween 60 twice weekly for 15 weeks. Mice photographed in 19th week of experiment.

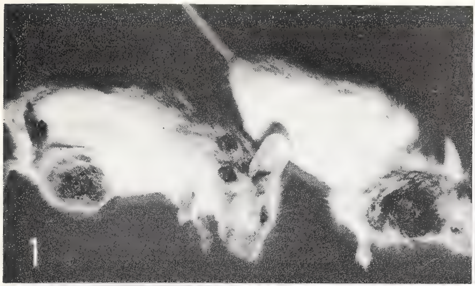
FIGURE 2.—Tumor seen in figure 1, bisected to show superficial scab, central necrosis, and peripheral rim of malignant cells characteristic of large squamous-cell carcinomas.

FIGURE 3.—Keratinizing epidermal carcinoma in interscapular space, and subcutaneous fibrosarcoma in left dorsolumbar region: virgin SWR female mouse intubated twice weekly for 32 weeks with 0.25 mg MCA in 0.1 ml Tween 60.

FIGURE 4.—Low-power photomicrograph: margin of interscapular tumor shown in figure 3. Border of normal skin, *left*, is elevated and rounded by infiltration of malignant nodule composed of keratin-forming squamous-cell carcinoma within a subcutaneous lymphatic vessel. Slitlike remainder of lymphatic vessel is seen between nodule and overlying, proliferating base of large keratin-filled crater of primary tumor. Other lymphatic spaces in subcutaneous tissue, *lower right half.*

FIGURE 5.—Higher magnification of area indicated by *arrow* in figure 4, showing infiltration of malignant peg of squamous-cell carcinoma into dilated lymphatic vessel.

FIGURE 6.—C57BL/6 male mouse (*left*) received intragastric intubations of 0.15 ml 2 percent DMBA twice weekly for 2 weeks. Photograph, taken 1 week after 4th intubation, shows marked generalized edema, compared with control mouse on *right.*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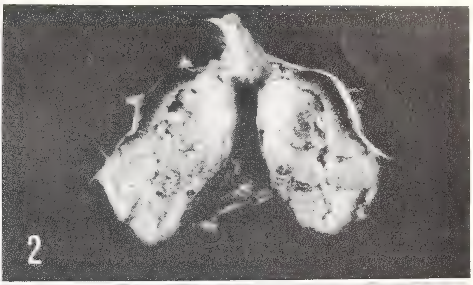
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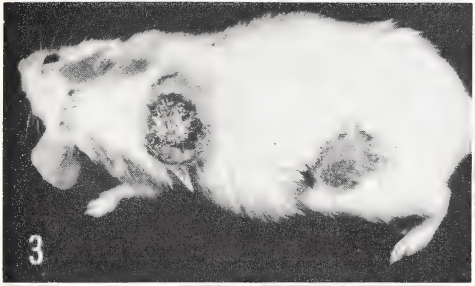
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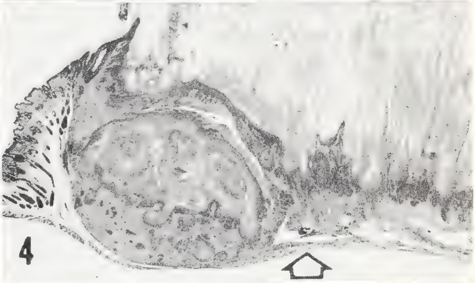
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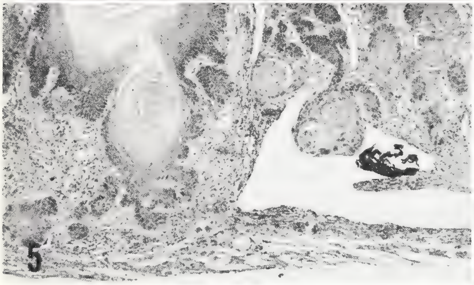
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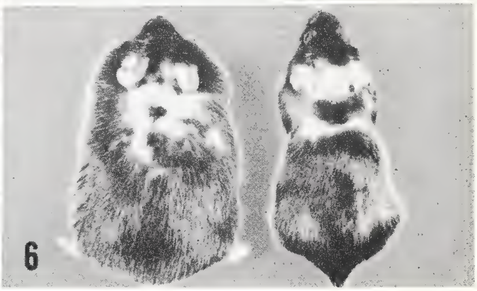
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Plate 65

Figure 7.—Marginal area of keratinizing carcinoma of forestomach, in mouse intubated repeatedly with BP. Neoplasm has extended through all coats of the stomach, after arising in region of limiting ridge. Hyperkeratotic lining and muscle layers of forestomach are seen at *right;* extension of the tumor under, and into glandular mucosa is at *left* of primary tumor mass.

Figure 8.—Peripheral portion of a malignant peg which invaded liver by direct extension, from a forestomach carcinoma induced by intragastric intubation of BP.

Figure 9.—Adhesions resulting from nonsuppurative visceral peritonitis and mesenteritis, after repeated intubation of 0.1 ml 2 percent MCA in Tween 60. In addition to firm adhesions binding kidney, liver, adrenal, and peritoneal wall, the intestines were completely immobilized by adhesions to other organs, and between adjacent loops.

Plate 66

Figures 10, 11, and 12.—Gross and microscopic characteristics of a dermal sarcoma, in a C3HeB male intubated twice weekly, for 29 weeks, with 0.1 ml 0.5 percent MCA in Tween 60. Portion of eroded epithelium, seen grossly in figure 10, is seen microscopically in *upper right* portion of figure 12; also remnants of hair follicles the bulk of which were engulfed by sarcoma.

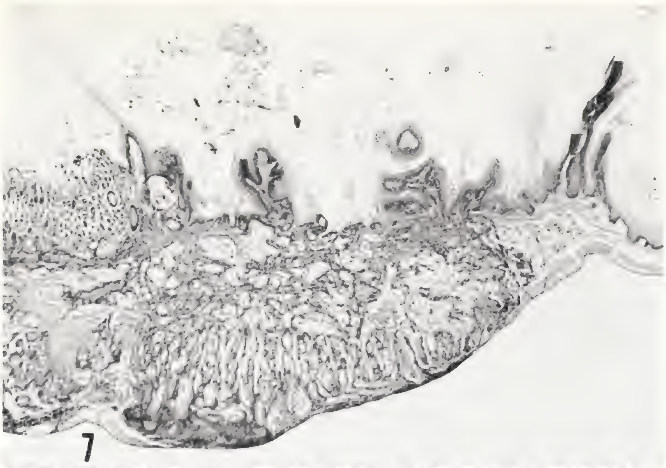
Figure 13.—Multicentric sebaceous-gland adenomas and adenocarcinomas on ventral abdominal and thoracic skin, and multiple primary carcinomas: SWR virgin female intubated twice a week for 40 weeks with 0.25 percent MCA in Tween 60. Carcinoma of right ear canal near right forepaw, a keratinizing squamous-cell carcinoma in left thoracocervical region, and a mammary carcinoma between right hind limb and tail.

Figure 14.—Low-power photomicrograph showing sebaceous-gland adenoma under an acanthotic epithelium, adjacent to a mammary cystadenocarcinoma (*right*); same mouse seen in figure 13.

Plate 67

Figure 15.—Sarcoma of mesentery with infiltration of a mesenteric vein: SWR female intubated with 1 percent MCA for 25 weeks, before it became moribund.

Figure 16.—Same case as above: showing mesenteric artery, its sheath infiltrated by tumor cells, piercing muscularis of small intestine in *upper left* quadrant of photograph. Tumor cells infiltrate submucosa along the course of artery to *left,* but not to *right* where arterial branches are absent. Infiltration of sarcoma into a mesenteric lymphatic is seen in *upper right* quadrant.



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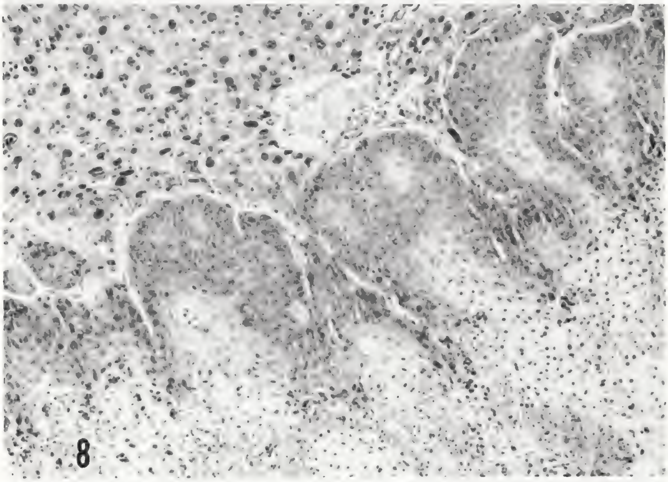

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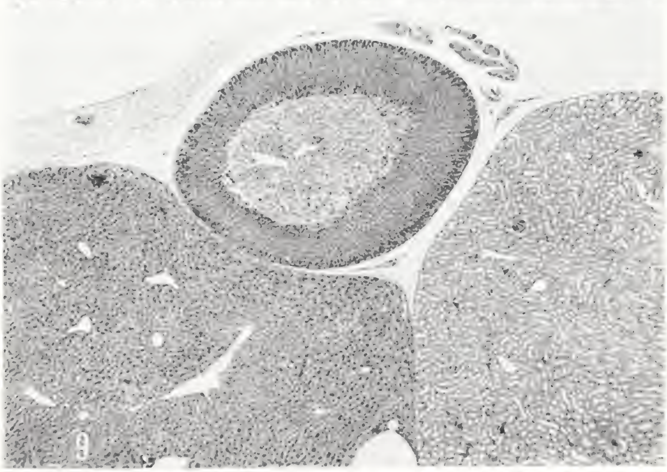

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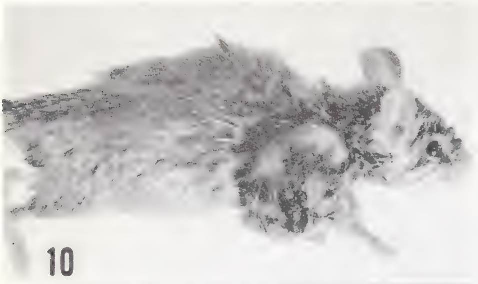

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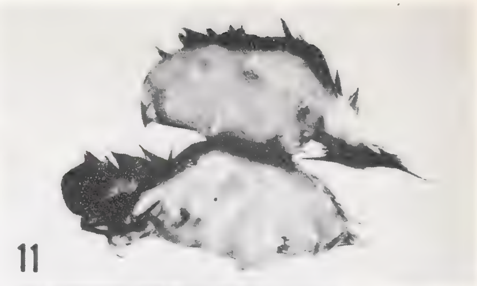

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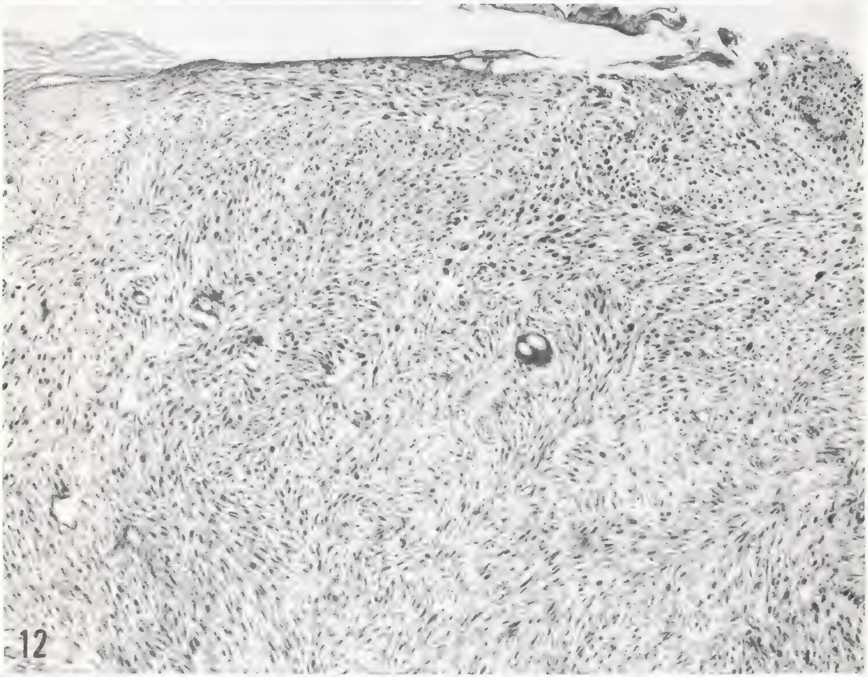

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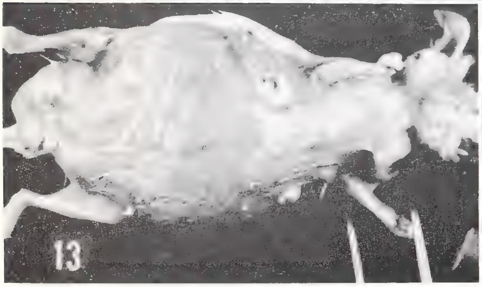

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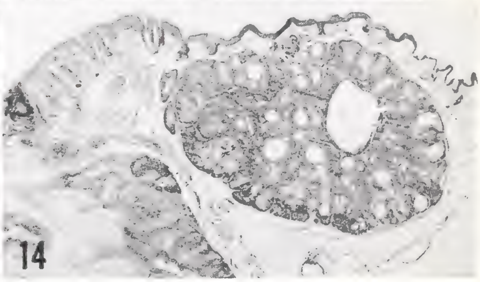

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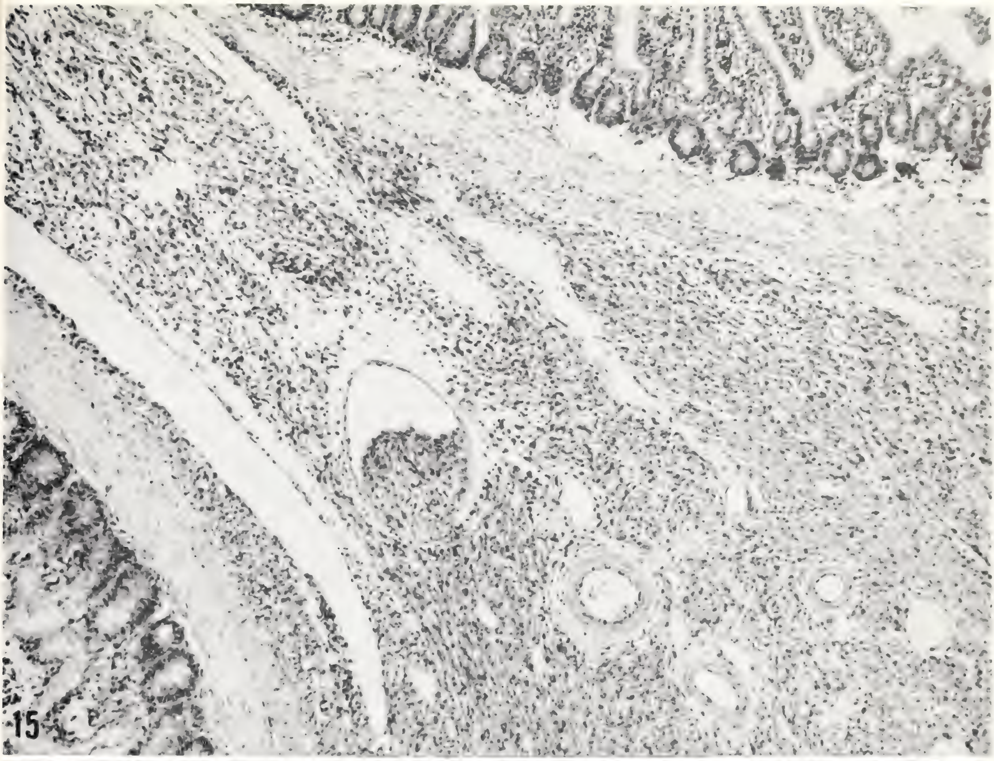
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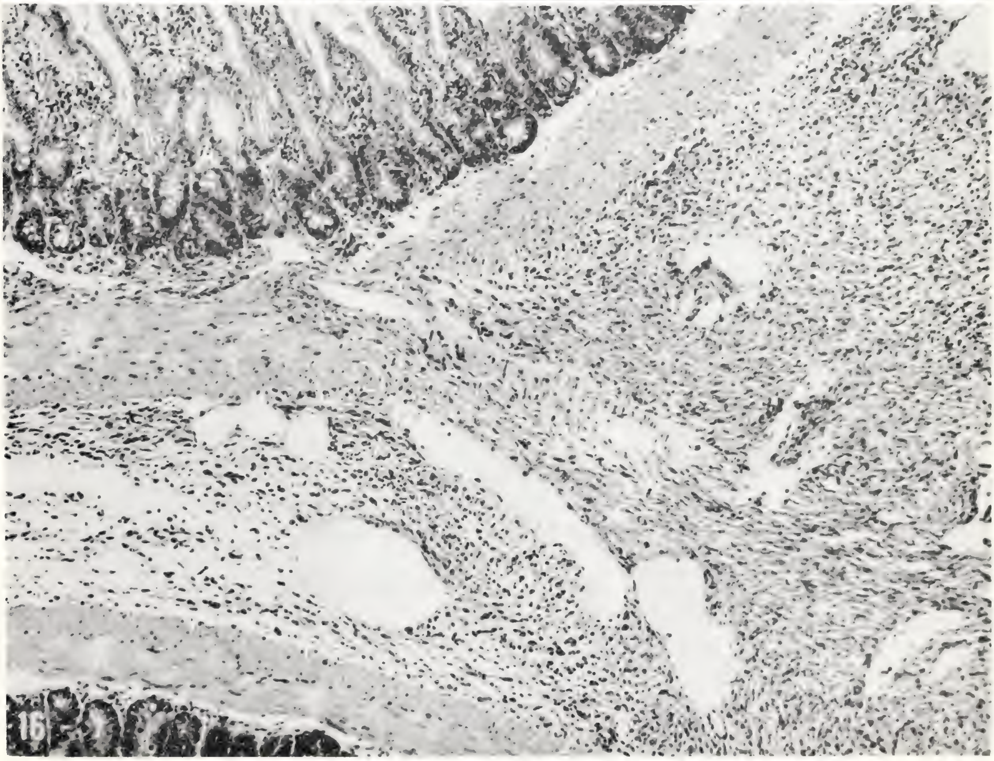
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AN EARLY TEST FOR POSSIBLE SKIN CARCINOGENS[1]

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SUMMARY

A tetrazolium reduction method revealed increased deposits of formazan in hairless mouse epidermis the first 2 days after a single application of 4 carcinogenic substances, whereas an initial decrease in formazan deposition followed the application of noncarcinogens. A blind test with 21 different substances revealed a complete correlation between carcinogenic activity and increased deposits of formazan. The method can be used as a quick test for possible carcinogenic activity of chemical compounds.—NCI Monogr. No. 10: 633–637, 1963.

THE CLASSICAL method of determining the skin carcinogenic potency of chemical substances is to administer single or repeated doses of the compound to a series of laboratory animals and observe the possible development of tumors. Such a procedure is time-consuming but, performed on a sufficiently large scale, gives a final answer.

With the increasing environmental exposure to new chemicals, there is clearly a need for quicker tests for possible carcinogenic activity. Among tests used during the last 5 years, we can mention "the sebaceous gland test" (*1*), "test d'hyperplasie" (*2*), and "the Newt test" (*3*). All these are based on judgment of histological alterations. The disappearance of the sebaceous glands and the development of hyperplasia are used as indicators of carcinogenic potency. Even if much information can be obtained by these tests, they are not specific and they cannot be performed quickly.

Because we were interested in the functional state of the mitochondria after a single surface application of carcinogenic substances, we decided to study this by means of a tetrazolium reduction method. Triphenyltetrazolium chloride is a colorless, water-soluble salt that can act as an electron acceptor. It is thereby reduced to a red, water-insoluble formazan. By incubation of tissues in a solution of tetrazolium, the activity of the energy-generating processes in the cells reduces tetrazolium to formazan. The amount of formazan per mg dry weight can be measured photometrically after extraction with acetone.

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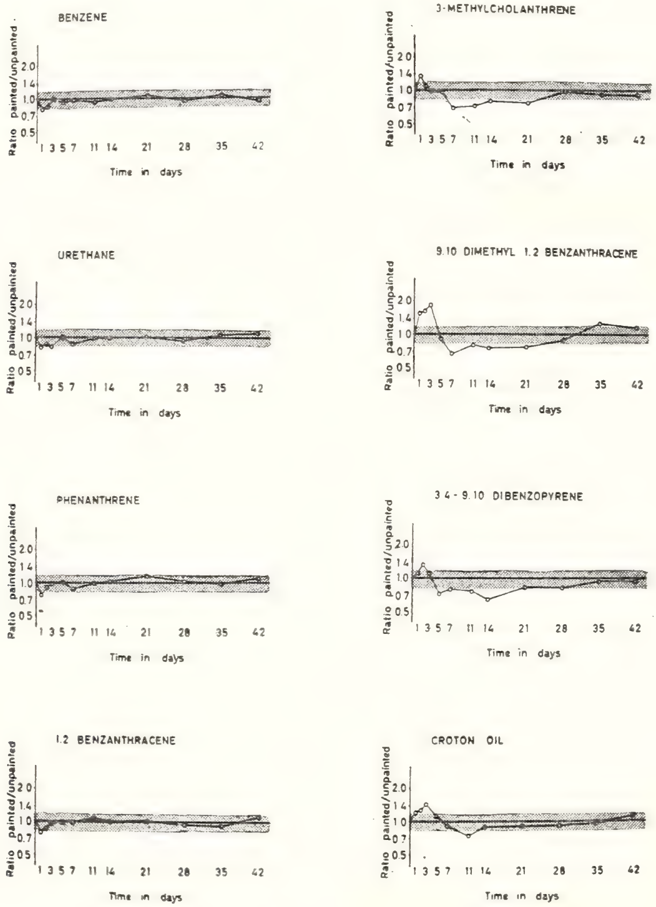
[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6-11, 1962.

[2] The author wishes to express his sincere thanks to the head of the Institute, Professor Leiv Kreyberg, for the excellent working facilities, and to Professor Lorentz Eldjarn for his cooperation with the blind test.

Different carcinogenic and noncarcinogenic substances were applied as a standard, single dose in benzene solution, by means of a special pair of forceps. At different time intervals after the application, groups of animals were killed and the skin immediately flayed off. The skin was then stretched on a frame and immediately incubated at 37° C for 1 hour in a solution of 1 percent 2,3,5-triphenyl-2*H*-tetrazolium chloride (E. Merck, A. G. Darmstadt) in M/15 phosphate buffer *p*H 7.2 in complete darkness and with no specific substrate added. The incubation was undertaken under aerobic conditions. After 1 hour the skin specimens had become deep red. They were then transferred to a bath containing 0.1 percent acetic acid in distilled water at a temperature of +4° C and kept there in the darkness for 24 hours. Thereafter it was possible to separate



TEXT-FIGURE 1.—Reaction of epidermis to a single application of different carcinogenic and noncarcinogenic compounds during first 42 days after application. *Dotted area* indicates ±2 standard errors of the method.

the epidermis from the corium with a blunt knife. A small piece of epidermis from the painted area was placed in one bottle and a piece of epidermis from a corresponding, nonpainted area of the same skin in another bottle. The red formazan was extracted overnight into airtight bottles with 3 ml acetone and kept in darkness. The optical densities of the different solutions were read with a Hilger spectrophotometer at 4800 A; distilled water was used as a blank. The small pieces of epidermis were then dried and weighed, and the extinction value per mg dry epidermis was calculated. The ratio between the value from the painted epidermis and the corresponding value from the unpainted epidermis from the same animal was then calculated and used as a relative measure of the amount of formazan deposited per hour in the painted epidermis.

A control study including skin specimens from 100 mice was first undertaken. The result showed that the method was sufficiently reliable when groups of 8 mice were used at each observation point. A new control study involving mice which were sham treated (only kept in the forceps) was then performed. The result showed that the effect of the sham treatment was negligible. An amount of 0.005 ml of the following noncarcinogenic solutions was then applied: benzene alone, 0.5 percent urethan in benzene, 0.5 percent phenanthrene in benzene, and 0.5 percent benz[*a*]anthracene in benzene. The resluts showed that the reaction of the epidermis to the application of benzene and of the noncarcinogenic substances was characterized by an initial slight decrease in the rate of formazan deposition (text-fig. 1).

The reaction of the epidermis to the application of the following carcinogenic solutions was then measured: 1.0 percent 3-methylcholanthrene, 0.5 percent 9,10-dimethyl-1,2-benzanthracene,[3] 0.16 percent 3,4-9,10-dibenzpyrene,[4] and 0.5 percent croton oil. All substances were dissolved in benzene. The results demonstrated (text-fig. 1) that the reaction of the epidermis to these 4 carcinogens was characterized by an initial rise in the deposits of formazan, followed by a period of decreased deposition. For a more detailed description of the method and the results, reference is made to a previous paper (*4*).

As the investigations seemed to allow the conclusion that an initial increase in the rate of formazan deposition in epidermis was a specific effect of a topical surface application of a carcinogenic substance, the validity of such an interpretation was tested with the following "blind test":

1. Twenty different compounds were dissolved in benzene in the concentration 3.73×10^{-2} M (this is the molarity of a 1.0 percent solution of 3-methylcholanthrene in benzene). Liquid compounds were used in the concentration 1.0 ml per 99.0 ml benzene. The preparation of the solutions was done by Klinisk Kjemisk Sentrallaboratorium, and the different specimens were labeled A, B, C, etc. The contents of the bottles were unknown to me until all the experiments were finished. After the blind test had started, a new substance (the 21st) was added. This was β-propiolactone, which was labeled U and was tested as a "known" substance.

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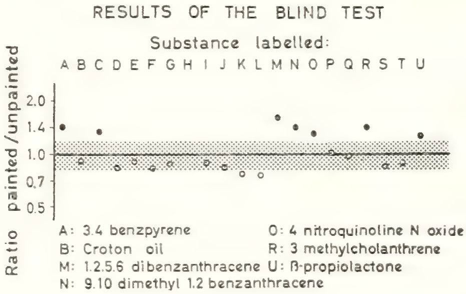
[3] *Chemical Abstracts'* nomenclature: 7,12-dimethylbenz[*a*]anthracene.

[4] *Chemical Abstracts'* nomenclature: dibenzo[*a,i*]pyrene.

2. Initially 16 mice (8 males and 8 females) were used for each test substance.
3. The standard tetrazolium method, as previously described, was used. The amount of formazan deposited in the treated epidermis was measured in 4 males and 4 females on the 1st day after application and in 4 males and 4 females on the 2d day. The mean value from these 16 mice was considered the result of the blind test.
4. As +2 standard deviations of the method with 8 mice in each group give a value of 0.180, the following interpretations of the results were outlined beforehand:
   a. Values of 1.2 and above should indicate a substance with carcinogenic potency.
   b. Values below 1.0 should indicate a substance without carcinogenic potency.
   c. Values between 1.0 and 1.2 should indicate a "doubtful result," and the test should be repeated and the result calculated as the mean of 32 instead of 16 mice. This happened 3 times (*see* table 1).
   d. If some of the substances induce necrosis and ulceration of the epidermis during the first 2 days after application, the test should be repeated with half the initial concentration. This happened once during my experiments, with substance labeled O. The result for this substance is thus obtained with a concentration 1.87×10^{-2} M.

The results of the blind test are shown in table 1 and text-figure 2.



TEXT-FIGURE 2.—Results of the blind test. *Dotted area* indicates ±2 standard errors of the method.

A complete correlation between carcinogenic activity and increased values of formazan deposition was found. It can thus be concluded that this method may be of value as a test for substances with possible carcinogenic potency.

It is difficult to draw a conclusion about the detailed mechanism involved in the reaction. If cells are not heavily damaged, there exists a rough quantitative correlation between the amount of formazan deposited per mg dry tissue per hour and the metabolic activity (in terms of Q_{O_2}) of this tissue. If cells are heavily damaged, however, a qualitatively new situation arises, with no correlation between formazan deposition and cellular respiration. Actually, investigations of the oxygen consumption of epidermal cells after a single application of 3-methylcholanthrene have revealed that during the initial period of increased formazan deposits there is also a significant *reduction* of respiration (*4*). This points to a damage of the mitochondria, perhaps an increased permeability of the mitochondrial membrane combined with blocking, and/or uncoupling of the respiratory chain.

TABLE 1.—Results of the blind test

Blind test				List of answers	
Compound	Number of mice	Arithmetic mean	SD of the mean	Compound	Skin carcinogenic potency
H	16	0. 682	0. 07	1,2-Benzanthracene*	
L	16	0. 754	0. 11	Pyrene	—
K	16	0. 770	0. 07	*d*-Limonene	—
F	16	0. 825	0. 12	1 Stearyl-2,3-polyoxyethylene	—
				monoglyceride	—
D	16	0. 827	0. 12	Naphthalene	—
J	16	0. 838	0. 09	3-Methyl-1,2-benzanthracene†	—
S	16	0. 848	0. 06	Oil of turpentine	—
G	16	0. 880	0. 13	Fluoranthene	—
T	16	0. 881	0. 09	Cholesterol	—
I	16	0. 897	0. 09	Phenanthrene	—
E	32	0. 908	0. 07	Urethan	—
B	16	0. 909	0. 08	Anthracene	—
Q	32	0. 970	0. 05	1,2,6,7-Dibenzanthracene‡	—
P	32	1. 010	0. 08	1,2,3,4,Dibenzanthracene§	—
U	16	1. 265	0. 15	β-Propiolactone	+
O	16	1. 307	0. 12	4 Nitroquinoline *N*-oxide	+
C	16	1. 337	0. 11	Croton oil	+
N	16	1. 405	0. 12	9,10-Dimethyl-1,2-benzanthracene	+
A	16	1. 408	0. 08	3,4-Benzpyrene‖	+
R	16	1. 410	0. 09	3-Methylcholanthrene	+
M	16	1. 634	0. 12	1,2,5,6-Dibenzanthracene**	+

* *Chemical Abstracts'* nomenclature: benz[*a*]anthracene.

† *Chemical Abstracts'* nomenclature: 3-methylbenz[*a*]anthracene.

‡ *Chemical Abstracts'* nomenclature: benzo[*a*]naphthacene.

§ *Chemical Abstracts'* nomenclature: dibenz[*a,c*]anthracene.

‖ *Chemical Abstracts'* nomenclature: benzo[*a*]pyrene.

** *Chemical Abstracts'* nomenclature: dibenz[*a,h*]anthracene.

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PROBLEMS IN THE ASSAY OF CARCINOGENS [1]

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SUMMARY

Assay of the potency of carcinogens demands knowledge of the quantitative aspects of cancer development. In cancer induction by ultraviolet light, there is adequate experimental data on mice, and a quantitative model to describe the dose-response relationships, but extrapolation to man from experiments on animals is not feasible. Some of the difficulties of assay, which also apply to other carcinogens, are illustrated with this material. Chemical carcinogenesis involves factors that do not pertain to ultraviolet carcinogenesis, and attempts to formulate a model that can account for various sets of experimental data have not been altogether successful. The data on chemical carcinogenesis appear widely disparate so that, lacking a unifying quantitative model, application of any method of assay is hazardous. In this situation, the setting of tolerance levels may be dangerously misleading and is inadvisable.—NCI Monogr. No. 10: 639–647, 1963.

THE SKIN AS AN ASSAY SYSTEM FOR CARCINOGENS

SKIN OFFERS a good site for the study of carcinogenesis. The tumor is readily recognizable and the moment of its appearance is more accurately determined than for internal tumors; rate of growth, which is an important characteristic, can also be measured readily. But the use of skin as an assay system presents difficulties, many of which are also encountered with other tissues or organs. One may ask, first of all, just what it is we want to assay. Presumably it is the carcinogenicity of a given agent in relation to man, but we are limited, as a rule, to attempts to measure this on some experimental animal.

Let us take the example of cancer induction by ultraviolet radiation. We have a good deal of interpretable quantitative data and an adequate model to describe carcinogenesis by this agent in the skin of mice (*1*). One would like to know, if possible, how to determine from such data what doses might be expected to produce cancer of the skin in man, whether from sunlight or from some artificial source. It seems at first glance that this should be easy, but when one comes to grips with the problem he finds considerable difficulty. The optical properties of the skin of the mouse or rat are quantitatively very different from those of the skin of man. In the former, the epidermis, particularly the corneum, is thinner on most areas of the body, and thus the radiation can penetrate to, and act upon, deeper tissues. Text-figure 1 permits a comparison of the amounts of ultraviolet light of various wavelengths transmitted by the epidermis of the mouse and of man. It is seen that very little of the radiation shorter than 0.32 μ, which causes sunburn and inducesc ancer, reaches the human dermis, and, correspondingly, those cancers of man that most likely may be attributed to sunlight are carcinomas arising in epidermal tissue, whether of basal-cell or squamous-cell type. On the other hand, in a long series of cancers induced in the ear skin of albino mice by radiation from an intermediate pressure mercury arc, sarcomas predominated over carcinomas of the epidermis (*1*, p. 201). This again can be largely explained on the basis of the penetration of this radiation into the mouse skin, a large fraction of the radiation from this source passing through the epidermis to be absorbed in the dermis, as shown in text-figure 1. Text-figure 2, which illustrates some of the relationships concerned, shows how much the situations differ for carcinogenesis by sunlight in man, and for carcinogenesis in the mouse by radiation from the intermediate pressure arc. These differences make any close quantitative comparison infeasible.

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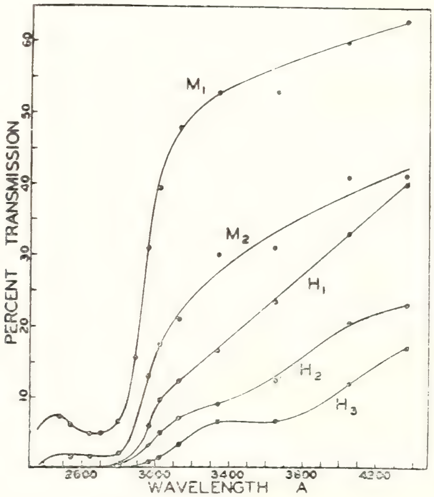
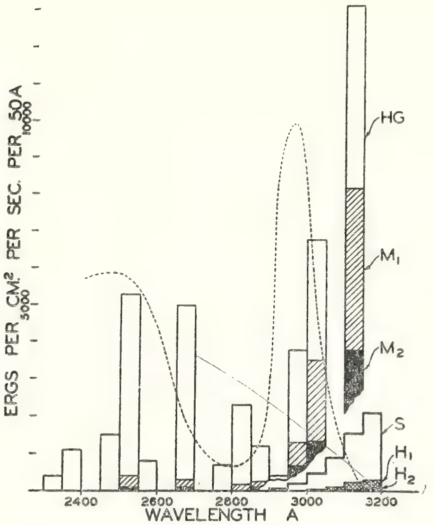
[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

With another type of source, the low-pressure mercury arc, which emits most of its carcinogenic radiation at wavelength 0.2537 μ, Kelner and Taft (*3*) produced only carcinomas. This wavelength is strongly absorbed in



Text-figure 1.—Transmission of human and albino mouse epidermis. M_1: from ear of normal young mouse; M_2: from ear of irradiated mouse; H_1: from untanned volar surface of human forearm; H_2: from slightly tanned volar surface of human forearm; H_3: from heavily tanned dorsal surface of human forearm. H_2 and H_3 were from the same person. From Kirby-Smith, Blum, and Grady (*2*).

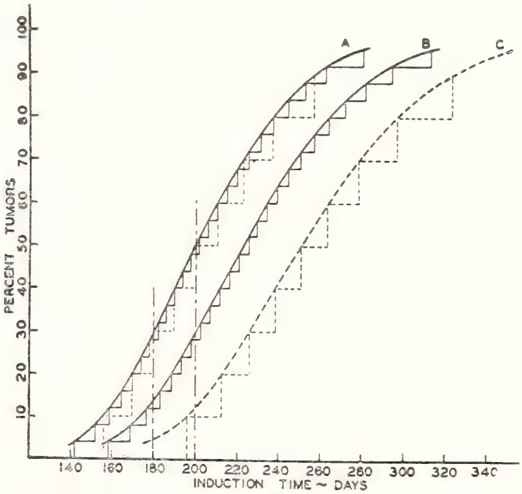


Text-figure 2.—Energy relationships in mouse skin exposed to an intermediate pressure mercury arc and human skin exposed to sunlight. HG: energy of the intermediate pressure mercury arc; in most cases the 50 A represented in the rectangle includes only one line. M_1 and M_2 represent, respectively, transmissions of normal and irradiated mouse epidermis; S represents energy of sunlight with clear, dry air mass and sun at zenith. H_1 and H_2 represent, respectively, transmissions of untanned and tanned human epidermis. *Dotted line* is the action spectrum for the erythema of sunburn. From Kirby-Smith, Blum, and Grady (*2*).

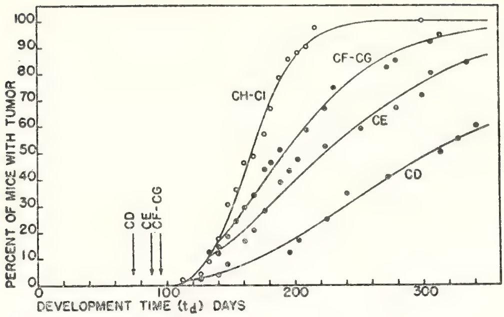
the epidermis, little passing through to the dermis, as may be seen in text-figure 1. The findings of Kelner and Taft are in line with what we know of the optical characteristics of mouse skin. Perhaps then, the carcinogenic action of the radiation from a low-pressure mercury arc on the mouse ear is more nearly comparable with the carcinogenic action of sunlight on human skin, though one would hesitate to say just how close. Of course, there are other areas of mouse or rat skin that may more nearly resemble that of man—the mouse tail has, for instance, a pretty thick corneum. For any close comparison we should consider other differences among types of skin. The epidermis of the mouse ear, for example, has a very thin layer of viable cells; before the skin has been irradiated this is only about two cells thick, and basal-cell and squamous-cell layers cannot be clearly distinguished as in human skin. It would seem very difficult to carry out a quantitative assay that would allow us to relate dosage of ultraviolet light on experimental animals to the dosage of ultraviolet light carcinogenic to man.

There are more general problems of assay that may be illustrated with studies of carcinogenesis by ultraviolet light. The time from first exposure to a carcinogenic agent to the appearance of a tumor of a given volume is a common, and perhaps the best available, measure of carcinogenicity. The tumor is usually growing so rapidly at the time it appears—it may be doubling its volume every few days—that there is no important experimental error introduced by choosing this as an endpoint. The time to tumor appearance is sometimes referred to as the "latent period," an obvious misnomer since the tumor must be growing during at least a considerable part of this time. The term "induction time," which is sometimes used, meets the same objection. I prefer to use *development time*—ultraviolet light carcinogenesis is clearly continuous throughout this time and the best explanation seems to be that something is growing from the time of the first dose (*4*). I do not want to quibble about names, however, but only wish to point out that at least a considerable part of the time to tumor appearance is occupied by growth, and that in a general way any quantitative measurement of carcinogenesis is a function of growth rate and hence has a time component. Disregard of the quantitative character of growth by cell proliferation may lead to erroneous conclusions, whether one is concerned with theoretical aspects of cancer or with the more practical problems of assay (*1*, chapt. 13).

A fairly common practice, and one which has grave weaknesses, is to dose a population of animals with a carcinogen, wait about 6 months, examine for tumors, and report the percentage of animals showing them. Such a measurement too has a time component, but this may be overlooked with this method of measurement. Text-figure 3, based on studies with ultraviolet radiation, in which the dose has been repeated over and over at regular intervals until the tumor appears, illustrates some weaknesses of this method. The percentage of mice with tumor is



Text-figure 3.—Quantitative relationships in carcinogenesis by ultraviolet light. The curves, based on a large number of experiments, indicate the variation of the development time (labeled induction time), with percent incidence of tumors in a population of albino mice. Curves A and B indicate the limit of significance (at the 0.02 level) for 2 groups of 25 animals each, when the development time at 50 percent incidence is 200 days. Curves A and C indicate the limit of significance for 2 groups of 10 animals each. The "steps" represent individual animals.



TEXT-FIGURE 4.—Delay of appearance of tumors as a result of discontinued dosage. The individual dose was the same in all cases. Curve CH-Cl: doses continued until tumor appearance. Curves CF-CG, CE, CD: dosage stopped at times indicated by *arrows* labeled with corresponding letters. From Blum (*5*).

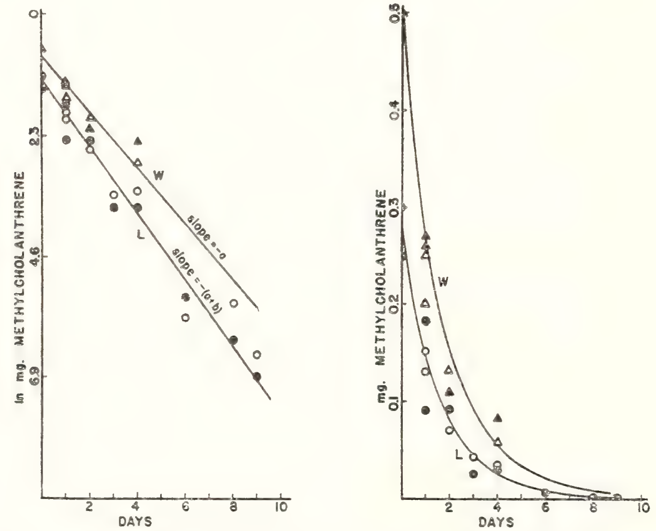
plotted against the development time. Let us consider that curves A and C represent 2 different experiments with different doses of ultraviolet light used. If we compare these 2 curves at 180 days we see that in curve A about 30 percent of the animals have tumors and in curve C about 4 percent, a ratio of 7.5. If we examine at 250 days, however, we see that in A about 50 percent of the animals have tumors and 30 percent in C, a ratio of only 1.7. The results may thus appear very different if we make our observations at different times after the first dose of carcinogen.

It seems better practice to observe the course of the appearance of tumors within a population—which can be very easily done with skin tumors—and to base our comparisons of carcinogenicity on the time to appearance of tumors in about 50 percent of the population of animals. The curves in text-figure 3 illustrate another important point with regard to assay. Curves A and B represent the difference that might be expected once in 20 times if a population of 25 animals were used, and times to reach a given percentage of tumors were compared. It is apparent that the error might be quite large, but if only 10 animals were used in the group, as represented by curves A and C, the accuracy must be very low indeed.

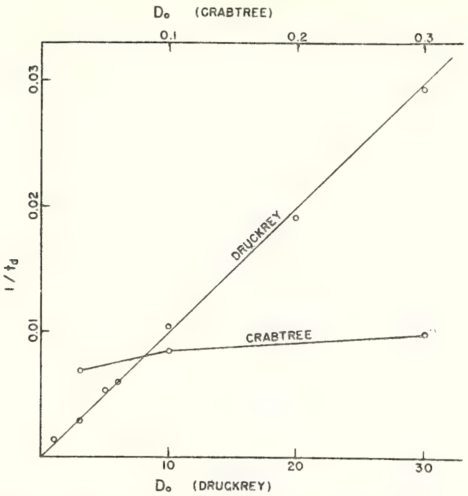
Another important point is illustrated in text-figure 4, which shows that to discontinue the dosage delays the appearance of tumors—the earlier the dosage is stopped the greater the delay. In the experiment described in text-figure 4, 100 percent of the mice developed tumors when the dosage was continued to time of tumor appearance, but when the dosage was discontinued the highest tumor incidence reached was limited to lower percentage values by deaths from causes not related to the cancer. Thus, the percentage of tumors reached is not an appropriate measure of carcinogenicity when the dosage is stopped early, nor would the mean development time be, for that matter, except that we have a quantitative model that describes this situation (*see 1*). In experiments with chemical carcinogens it is common practice to give only 1 dose, which would be comparable to discontinued dosage with ultraviolet light; in such case the percentage of animals with tumor has little meaning as an assay. Quantitative studies with repeated doses of chemical carcinogens, of which only a few have been made, could give us important information regarding both assay and the mechanism of carcinogenesis—particularly if the effect of interval between doses and interruption of dosage were examined.

Dosage relationships for chemical carcinogens are very different from those for ultraviolet light. In the latter one turns on the light, lets it run at a given dose rate for a given time, and then turns it off again—we have what may be called a "rectangular" dosage. With a chemical carcinogen, on the other hand, no matter where or how applied, the agent persists in the body, and in the various tissues for some time and disappears gradually according to a characteristic die-away curve, as illustrated in text-figure 5. Furthermore, the relationship between carcinogen introduced into the body and that which has reacted with the tissue at the site of tumor induction is unknown, and it must be expected to vary according to the manner of introduction of the carcinogen as well as with its chemical constitution. Under such conditions, it does not seem surprising that the dose-response relationships found experimentally for chemical carcinogenesis are different from those found for ultraviolet light carcinogenesis.

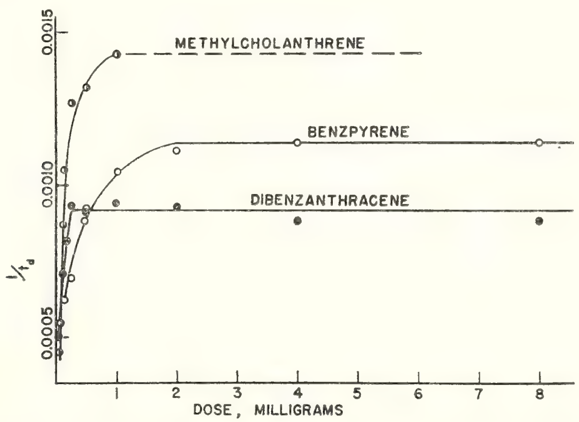
An idea of the wide variation in quantitative relationship that may be observed in chemical carcinogenesis is given in text-figures 6 and 7. The experiments were carried out by different investigators under quite



Text-figure 5.—Time course for loss of intravenously injected 3-methylcholanthrene from body of mouse: semilogarithmic plotting *on left;* arithmetic plotting *on right.* W: carcinogen in whole animal; L: carcinogen in lungs. Data from Lorenz and Shimkin (*6*).



Text-figure 6.—Dose-response relationship in experimental carcinogenesis under different experimental conditions. Druckrey (*7*) produced hepatomas in rats by daily oral administration of *N,N*-dimethyl-*p*-phenylazoaniline. Crabtree (*8*) produced papillomas of the skin of mice by biweekly painting with benzo[*a*]pyrene. The points represent the reciprocal ($1/t_d$) of the development time at the 50 percent tumor incidence level.



Text-figure 7.—Dose-response relationships for 3-methylcholanthrene, benzo[*a*]-pyrene, and dibenz[*a,h*]anthracene, injected into the axillae of mice. The points represent the reciprocal of the development time ($1/t_d$) at the 50 percent tumor incidence level (*9*).

different conditions. In text-figure 6 two experiments are illustrated in which the same dose of carcinogen was given at regular intervals until the appearance of the tumors; the reciprocal of the development time, symbolized as t_d, is plotted against the individual dose. In the experiments of Druckrey (*7*), hepatomas were induced in rats by feeding *N,N*-dimethyl-*p*-phenylazoaniline daily; these data give a good straight line that passes through the origin, which indicates that the time to tumor appearance is inversely proportional to the dose. The other data plotted in this text-figure are from an experiment by Crabtree (*8*) in which benzo[*a*]-pyrene was painted on the skin of mice; unlike Druckrey's data, these show no simple reciprocal relationship between dose and development time. The data plotted similarly in text-figure 7 are from Bryan and Shimkin (*9*)[3] who injected single doses of the 3 carcinogens, 3-methylcholanthrene, benzo[*a*]pyrene, and dibenz[*a,h*]anthracene into the axillae of mice. Again we see that reciprocity between dose and development time is far from satisfied. An attempt has been made to formulate a quantitative model that would explain the apparent discrepancies between these various data, taking into account the factors mentioned, but without complete success. This search has been useful, however, in bringing recognition of the complexity of the factors concerned.

In text-figure 7, for benzpyrene and dibenzanthracene, the development time becomes constant above a certain dose, *i.e.*, increase in the dose of methylcholanthrene would show the same thing if high enough doses had been given, and there is evidence for this in the data of Cramer and Stowell (*10*). It is of particular interest that we find the same thing in carcinogenesis by ultraviolet light (*1*, fig. 41). It may well be that if sufficient data were available such a maximum effective dose level would be found characteristic of carcinogenesis by all agents. If that should be the case, we might have a method for comparing the effectiveness of these agents. It might be practical to take benzpyrene as a standard and then relate to it the minimum tumor development time for other carcinogens; if it worked out, time could be saved by working where the time to appearance of tumors is shortest, and at the same time where the variance is least. And, what is most important, natural mortality would be avoided to a great extent. But this may be going too far in speculation with the paucity of data available.

May I point out again the wide differences in dose-response relationship that have been obtained with different methods of experimentation with chemical carcinogens (*e.g.*, text-figs. 6 and 7). Until we have some explanation of these differences in terms of a quantitative model—which may be tantamount to our finding an explanation of the cancer mechanism—we are hardly in a position to set up any definitive assay method of carcinogenic potency. Toward this end, I would like to make a plea for more experiments with chemical carcinogens, carefully designed and carried out, to reveal the basic relationships between dose and carcinogenic response. Studies of the effect of doses repeated with various intervals and arrangements, such as have been carried out with ultraviolet light, could be particularly enlightening. I think that only with such studies can we hope to develop a rational approach to the assay of carcinogenicity.

The successful extrapolation from experiments on animals to man seems vague and distant. The experimental data we have—regardless of any model or hypothesis—indicate the futility of trying to set minimum tolerance levels for experimental animals, let alone for man. It would seem that any substance shown to produce cancer in experimental animals *in any dose* must be suspect and not to be recommended for human use if this is at all feasible. Tolerance levels set arbitrarily, or on the basis of uncertain assays, may only encourage a false notion of security.

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[3] Dr. Bryan and Dr. Shimkin have kindly made their original data available to me.

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TECHNIQUES AND METHODS IN STATISTICS AND EPIDEMIOLOGY OF SKIN CANCER[1]

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WITH AN ADDENDUM[1] *by* MORTON L. LEVIN, M.D., Department of Epidemiology, Roswell Park Memorial Institute, Buffalo, New York

SUMMARY

Techniques for the evaluation of comparative studies in skin cancer are discussed. Difficulties in the assessment of the malignancy of some tumors are given. Relative ratios to compare distributions by site and histological pattern may, with precautions, be used. Mortality rates for skin cancer are of no value because of the high rate of cure, but morbidity rates can be used satisfactorily. Reports on the geographical pathology of skin cancer, in addition to the over-all figures, should include information which will permit comparison of site and histological type. Studies of precancerous lesions and etiological factors should give data on the proportion of rural and urban patients, nature of occupation, and exposure to known or possible carcinogens.—NCI Monogr. No. 10: 649–656, 1963.

THE BASIC techniques for study of the geographical pathology of cancer of the skin do not differ from those used for study of cancers at other sites and are adequately covered in standard texts. However, certain additional precautions are necessary in evaluating comparative studies in skin cancer due to the high rate of cure of many tumors. Further, there are also difficulties in assessing the malignancy of some tumors even by histological examination.

RATIO STUDIES

In many countries the only available reports on the frequency of skin cancer are relative ratio studies from general hospitals or individual departments, the number of skin cancers being reported as the proportion of all cancers or of all patients admitted. Little reliance can be put on such relative frequencies which do not necessarily reflect incidence. Since tumors of the skin are readily accessible to clinical observation and available for biopsy, the ratio of skin cancer to other cancers tends to be unduly high where medical facilities are limited. Hospital admissions are also highly biased, since many patients with skin cancer will not be admitted and are treated as outpatients. Thus it is not improbable that the high ratios reported in parts of Africa are the result of an artifact (*1*).

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[1] Presented at The Conference on Biology of Cutaneous Cancer, Philadelphia, Penna., April 6–11, 1962.

However, it may be possible to use relative ratios to compare distribution by site and histological pattern if suitable precautions are taken. For example, marked differences between the relative frequencies of squamous and basal-cell carcinoma were demonstrated in the South African white and Bantu (*1–3*); these were later confirmed by incidence studies. The distinctive ratios of squamous to basal-cell carcinoma in the deeply pigmented races are now well recognized. Such demonstration of abnormal ratios may provide a useful stimulus for further incidence studies.

MORTALITY RATES

Mortality rates for cancer of the skin are of no value due to the high rate of cure of most skin cancers, and they may merely reflect the quality of medical services and the patients' attitude toward them. The death rates from cancer of the skin in the white and nonwhite races in the United States differ only slightly (*4*), but marked differences are seen in the morbidity rates (*5*). Autopsy material is of no value for the same reasons that the study of mortality rates is unreliable.

MORBIDITY RATES

The study of morbidity rates is the only satisfactory method for the presentation of data on skin neoplasms. Furthermore, due to the ease of diagnosis, reasonable rates may be determined for skin cancer in areas where total cancer rates or rates for the inaccessible sites may not be obtainable.

CLASSIFICATION OF SKIN TUMORS BY HISTOLOGICAL TYPE AND SITE

All reports on the geographical pathology of cancer of the skin, in addition to presenting the over-all figures, should also include information which will permit comparison according to site and histological type. For example, the over-all incidence of cutaneous, epithelial neoplasms in the South African Bantu is very low compared to that of the white population of the United States. However, the incidence of malignant melanoma is similar in the two groups and this fact would be obscured if only the over-all incidence was considered. Schrek (*6*) demonstrated that the difference in the incidence of skin cancer between the United States white and Negro was predominantly confined to the exposed skin, the frequency being similar on the unexposed surfaces.

Cancer of the orifices and external genitalia should also be reported separately, as in the international classification of disease.

Histological classification is also important in elucidating etiological factors since the major histological types vary in both etiology and biological behavior (*7–9*). Such a histological classification has proved of value in etiological studies in cancer of the lung (*10*) and liver (*11*). In addition to the major histological types, such as squamous-cell carcinoma, basal-cell carcinoma, etc., mesenchymal tumors such as Kaposi's hemangiosarcoma should be listed separately. It is also desirable that where a tumor is rare, this negative fact should be stated. For example, it was difficult in the South African study to compare the incidence of Kaposi's hemangiosarcoma in the Bantu with that in other races, due to lack of information on this tumor in the latter. An unusually common type of tumor in a geographical area should be given a separate classification.

Brodie's classification was originally introduced for squamous-cell carcinoma of the skin, but little data are available on variations in races with differing incidence. Further, such staging is highly subjective and comparisons by various observers offer difficulties, yet it is not improbable that variations indicating biological and etiological differences may be found in different races for the same type of tumor (*12*).

In addition, further data should be collected on the biological behavior of skin tumors in different population groups in regard to frequency of metastases, rate of growth, etc. Differences have been demonstrated in the frequency of metastases in skin carcinoma in males and females (*13*).

STUDY OF PRECANCEROUS LESIONS AND ETIOLOGICAL FACTORS

Pathological investigations of precancerous cutaneous lesions have been chiefly limited to the United States and Western Europe. More data on the epidemiological distribution of such lesions as nevi, solar dermatoses, etc., should be collected to determine their exact relationship to skin cancer in different races (*14*). If junctional nevi, for example, were invariable precursors of malignant melanoma, a correlation should be demonstrable in several countries between the incidence and site distribution of these nevi and malignant melanomas. Such studies present considerable technical difficulties, but attempts should be made to calculate prevalence rates by examination of autopsies, complete villages, troops, school children, etc. (*15*). Consideration should be given to the method of diagnosis since nevi may frequently be missed on superficial examination in deeply pigmented races. If autopsies are available, histological sections should be obtained to classify the type of pigmented lesions.

The incidence of solar dermatosis should also be determined in different population groups, possibly through the use of standardized histological preparations obtained at autopsies in general hospitals. Similar methods may be applied to other possible precancerous conditions such as hyperkeratosis, old injuries, focal depigmentation, etc. Studies of skin pigmentation should be presented in some detail with the use of standard anthropological techniques.

Until recently, environmental studies on skin carcinoma have been limited to relatively few areas and occupations. All studies of this type should give data on the proportion of rural and urban patients, nature of occupation, and exposure to known or to possible carcinogens such as ultraviolet light and arsenic ingestion. Every effort should be made to assess such information by the use of objective techniques including chemical analysis of the skin, use of ultraviolet exposure meters, etc.

The prevalence of albinism in a community should be established because albinos are highly susceptible to skin cancer and this group might prove suitable for comparative studies.

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ADDENDUM

EPIDEMIOLOGY IS the science of factors affecting the incidence of disease in populations. The methodological problems encountered in epidemiology and geographic pathology necessarily vary with the questions we are trying to answer. The primary emphasis at this meeting has been on studies of variations in the occurrence of skin cancer in different geographic and ethnic groups. In such studies the questions are: 1) How can we best determine whether real differences exist; 2) what are they; 3) to what extent do known etiological factors account for these differences; and 4) what other factors should be studied? These are broad questions; the methodology of any investigation will be determined by the nature and precision of the questions, the character of the population to be studied, and the facilities available for obtaining various types of information, such as census data and medical and diagnostic services.

Most of the studies in geographic pathology of cancer reported here or elsewhere have been concerned with the first 2 questions listed in the preceding paragraph, *i.e.*, differential frequency and the methods of studying this. Frequency of disease is measured directly by identification of new cases, all cases or deaths occurring in a specified time, and expressing these as a rate per unit of population, usually per 100,000. Pathologists who are interested in determining one or more of these measures of frequency must concern themselves with the methods of recording information on cases and deaths and with the methods of population enumeration and recording of census data. An indirect method of studying relative incidence of types and sites of cancer is to compare their distribution in a representative sample of cases from the populations compared—the ratio method.

RATES, RATIOS, AND PERCIVALL POTT'S OBSERVATION

Pott's observation obviously has special relevance to a discussion of the methodology of the epidemiology of skin cancer. It was the first observation that correctly identified the relation between an occupation, an environmental agent, and a particular form of cancer in man and which led to the first experimental demonstration of the carcinogenic effect of a chemical agent in animals. Since much of epidemiological methodology is statistical, it is startling to note that Pott's report contains no statistics, not even the number of cases of scrotal cancer he saw in chimney sweeps. His report (*1*) states ". . . everybody is acquainted with the disorders to which painters, plumbers, glaziers and the workers in white lead are liable; but there is a disease as peculiar to a certain set of people which has not, at least to my knowledge, been publicly noticed; I mean the chimney-sweepers' cancer. . . . It is a disease which makes its first appearance in the inferior part of the scrotum . . . other people have cancer of the same parts, and so have others beside lead-workers, the Poictu colic, and the consequent paralysis: but it is nevertheless a disease to which they are peculiarly liable; and so are chimney sweepers to the cancer of the scrotum and testicles. . . . The disease, in these people, seems to derive its origin from a lodgement of soot in the rugae of the scrotum . . . ." The rest of the approximately 800 word paper is concerned with diagnosis, clinical course, and treatment.

Since Pott cited no statistics, we may try to do this for him. In subsequent reports from St. Bartholomew's Hospital, where he worked, we find: "There was observed in St. Bartholomew's Hospital 29 cases of sweeps' cancer among a total of 39 scrotal cancer treated during 1880 to 1890 (*2*). This is evidently all the information Pott had and all he needed.

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> One wonders whether this information would be accepted today as a sufficient basis for some action if action were possible, important, and urgent. A modern instance is the recent (1961) outbreak in Germany, where within a short period 100 babies whose mothers had previously taken a new hypnotic, thalidomide, were born with the normally rare, congenital abnormality phocomelia (*3, 4*). This report was met with the following medical objections to any practical inference, *i.e.*, the sale of the drug: "The evidence against the drug is only statistical." "The relation has not been proven conclusively." "Thousands have taken it and delivered normal babies." "The drug may be the victim of a coincidence." "The drug has not yet been pinpointed as the sole cause."

★ ★ ★

To convert Pott's data, the right hand column may be filled in with other data which correspond to his knowledge that other cancers and diseases were less frequent in chimney sweeps than was scrotal cancer.

POTT'S DATA

I (*2*)

	Scrotal cancer		Other comparison groups	
Chimney sweeps	0.74	(*a*)	Very small	(*b*)
Others	0.26	(*c*)	Very large	(*d*)

In E. L. Kennaway's study (*5*), for 1911–12 in England and Wales, these values are:

	Scrotal cancer	Other cancer
Chimney sweeps	0.25	0.0018
Other	0.75	0.9982

$$\frac{ad}{bc}=185 \text{ and } \frac{a}{b}=139$$

This indicates that the intuitive, and methodologically incorrect, judgment, that the relative risk of occurrence of disease A in association with a characteristic is given by a comparison of the ratio of the proportions of persons with and without the characteristic in group A compared with other comparison groups, may be qualitatively correct when one of these proportions is very small. This seems to have been the basis for Pott's correct inference. The combination of a normally infrequent, but identifiable, characteristic occurring in a high proportion of cases of a rare disease provides the optimal conditions for recognition of an association from scanty and incomplete data.

The criticism must be considered that relative risks estimated from a comparison of proportions of cases, as in ratio studies in different ethnic and geographic populations, or in occupational groups compared to general populations, do not distinguish between differences arising from differences in absolute incidence of a disease and those arising from differences in incidence in relation to other diseases. In a ratio study of cancer A, in an occupational group, for example, we may find:

	Occupational group I	General population, group II
Cancer	0. 70	0. 10
Other cancer	0. 30	0. 90

The indicated risk of cancer A compared to that of other cancer is 31 times as great in group I as in group II, if we assume age and sex distributions have been considered. This may be due to an excess incidence of A in group I, 31 times that in group II, or to a reduction in the incidence of "other cancer" in group I to $\frac{1}{31}$ that in group II, or to a reduction of A to $\frac{1}{2}$, and of "other cancer" to $\frac{1}{62}$. These possibilities have led to the conclusion that since such data cannot be distinguished between the alternative explanations, they should always be followed by studies of absolute incidence in the 2 populations before any inferences can be made. This conclusion is sound statistically. However, it should be noted that any of the possible explanations of a markedly different ratio between 2 groups indicates an atypical situation. Where, for example, immediate decision regarding possible action is needed, we must consider the likelihood of alternative explanations, as well as their statistical possibility, before dismissing the excess absolute risk explanation until absolute incidence data are available. In some instances, the data for ratio comparisons permit also some minimum estimate of total incidence, so that the maximum probable reduction in the incidence of other cancer, and the extent to which this reduction could explain the ratio differences, may be estimated. It is not always essential to have rate studies before proceeding to etiological studies or before making some decision based on the probability of alternative explanations of the ratio data.

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RESOLUTIONS AND RECOMMENDATIONS OF THE CONFERENCE ON THE BIOLOGY OF CUTANEOUS CANCER

Geographic Pathology and Epidemiology:

In view of the general dearth of epidemiologic and pathologic studies on skin cancer suitable for comparative purposes in most parts of the world, this Committee recommends:

1. That morbidity studies should be organized in countries with sufficient facilities. Where these are not practical on a nation-wide base, they should be conducted on well-defined characteristic areas within the country concerned. Attention is drawn to the fact that mortality rates are of little value except for malignant melanoma.

2. It is desirable that a number of centers be set up to work in collaboration, both in the same and in separate countries.

3. The presentation of all data should be in accordance with recognized statistical procedures and standard international classifications and nomenclatures.

4. Materials should be classified by age, sex, ethnic group, and, in the case of immigrants, country of birth and duration of residence.

5. Wherever possible, all lesions should be biopsied and classified according to an agreed histopathologic classification.

6. An attempt should be made to collect from the populations-at-risk as many pertinent anthropological data as possible, including skin pigmentation, hair and eye color, texture and thickness of skin, and distribution of hair follicles, sweat glands, and pigmented spots according to standard anthropological and pathologic techniques.

7. Similar data should be given on sociological, dietary and cultural habits, including occupation, and known or suspected occupational hazards. Clothing and exposure of skin areas and the use of drugs and other similar substances for medicinal and other purposes should also be described.

8. Geophysical and meteorological data should include altitude, latitude, hours of sunlight, average day temperature, its intensity, rainfall and humidity, and where practicable, the intensity of ultraviolet radiation.

9. Where morbidity statistics of the kind outlined are not available we wish to emphasize that many useful data may be obtained from the collection of ratio studies, provided that proper precautions are taken to avoid bias, such as may occur, for instance, in data collected from individual hospitals and departments offering special treatment.

10. Studies should be conducted to determine the possible effects of specific environmental etiological factors in accordance with recognized statistical procedures, including adequate controls. Retrospective and prospective studies may prove most useful for this purpose. Individual studies that in themselves are inconclusive, may be of importance when considered in association with other studies with which they show consistent patterns.

11. Studies should be devised and carried out to establish the precise relationships between pigmented nevi and the so-called precancerous conditions, such as "keratosis," and identified cancers, including those of the internal oragns.

Pathology, Histochemistry, and Classification:

1. The Committee recommends that central laboratories be designated as reference and histopathologic registries 1) to assist in the standardization of histopathologic diagnosis, 2) to serve as a repository for histopathologic and clinical material of importance and interest, and 3) to advise on histochemical and other special histopathologic procedures appropriate to the description and the investigation of cutaneous tumors..

2. It is suggested that, insofar as possible, diagnosis of cutaneous tumors conform to classification by histopathologic type, anatomic site, and known environmental or genetic predisposing factors:

a. Histopathologic Types

1) Squamous-cell carcinomas (of skin exclusive of mucosa of lip, penis, scrotum, vulva)
    - i. Squamous-cell carcinoma
    - ii. Squamous-cell carcinoma arising in burns, ulcers, scars
    - iii. Senile keratosis with squamous-cell carcinoma
    - iv. Squamous-cell carcinoma arising in other precancerous dermatoses

2) Keratoacanthoma

3) Basal-cell carcinoma
    i. Basal-cell carcinoma
    ii. Superficial "multicentric" basal-cell carcinoma
    iii. Basal-cell carcinoma of hamartomatous origin
4) Adnexal carcinoma
    i. Of sweat gland origin
    ii. Of sebaceous gland origin
    iii. Unclassified (including basosquamous carcinoma)
5) Precancerous lesions and dermatoses
    i. Bowen's disease
    ii. Intraepidermal epithelioma of Jadassohn
    iii. Senile keratosis
    iv. Extramammary Paget's disease
    v. Erythroplasia of Queyrat
    vi. Leukoplakia of the vulva, penis, anus, lip
    vii. Others, as specified
6) Malignant melanoma
7) Sarcomas
    i. Kaposi's sarcoma
    ii. Dermatofibrosarcoma
    iii. Others, as specified
8) Lympho-reticular tumors and leukemia
    i. Leukemias, by type
    ii. Lymphosarcoma
    iii. Plasma-cell sarcoma
    iv. Reticulum-cell sarcoma
    v. Mycosis fungoides
    vi. Hodgkin's disease
    vii. Other lympho-reticular tumors

b. All Lesions Should Be Accurately Identified as to Anatomic Site With as Great Specificity as Possible

The use of general anatomic sites such as "face" and "lower extremity" are not satisfactory, for they are likely to conceal differences in distribution that are significant. Distinctions between the skin and mucous membranes of the lips, vulva, penis, scrotum, and anus should always be made.

c. Known Predisposing Exposures and Genetic Predispositions

1) Environment
    i. Radiation
        a. Actinic
        b. Ionizing
    ii. Chemical
        a. Occupational
        b. Therapeutic
        c. Others, as specified
    iii. Social (custom and habit)
    iv. Burns, scars, and ulcers
2) Predisposing genetic disorder
    i. Albinism
    ii. Xeroderma pigmentosum
    iii. Basal-cell hamartomas (basal-cell naevi).

Etiology:

1. Skin cancer has the largest number of identified etiologic agents for man. The extent to which known etiologic agents account for existing incidence should be fully explored.

2. Epidemiologic and demographic studies of the incidence, topographical distribution, sex and age ratio, skin pigmentation, and histologic type of precancerous and cancerous skin lesions should be conducted on population groups composed of members of different races and exposed to various natural and man-made environmental conditions.

3. A thorough analysis should be made of the possible factors underlying the apparent absence of carcinogenic response to therapeutically administered coal tar, mineral oil, and ultraviolet radiation.

4. The role of trauma in the development of malignant melanomas deserves critical analysis.

5. The development of standardized methods of measurement of solar ultraviolet radiation should be encouraged. The coordination and support of meteorologists should be obtained in the study of solar energy and ozone content of the atmosphere.

6. It is essential to study the differences in the histology and biochemistry of the skin of racial groups and groups of individuals with different degrees of pigmentation, with particular reference to keratinization and melanogenesis, and the effects produced on these and other constituents of the skin by ultraviolet radiation. Groups

deserving special attention are persons with albinism, vitiligo, and xeroderma pigmentosum. The belief that heavily pigmented races show less skin reaction to ionizing radiation should be investigated. A similar problem is presented by the reported difference in the response of skin of persons of light and dark pigmentation to chemical carcinogens.

7. The high incidence of skin cancer in an area of Southwest Taiwan having a high concentration of arsenic in the water supply has been reported at this conference. Such areas offer the opportunity for epidemiologic and preventive studies in arsenical cancer.

8. Since it will not be possible to eliminate from the environment all agents known or suspected to be carcinogenic, it obviously would be of great value to intensify the search for anticarcinogenic agents.

9. Investigations are indicated into the endogenous factors related to the natural refractoriness to cancer induction of certain animal species, including nonhuman primates.

10. Because of the persistent failure to induce arsenic cancer in various animal species it is desirable to study horses, in view of the high incidence of skin tumors in the horse and the frequent treatment of horses with arsenicals.

11. The frequency of development of squamous-cell carcinoma in association with certain chronic ulcers (tropical ulcers, burn scars, syphilitic ulcers, lupus vulgaris, and discharging sinuses) contrasts with its rarity in other forms of chronic ulceration, notably varicose ulcers. This needs further investigation with specific reference to the frequency of malignant change in these lesions and the duration of the ulceration prior to malignant change. Comparative study of all types of chronic ulcers is needed with comparison of cases with and without cancer. The relative importance of factors such as wound healing, microbial products, irradiation, chemical carcinogens, and nutrition in the genesis of these cancers should be investigated.

12. The clinical course of keratoacanthoma and the lesions described by Ferguson-Smith suggest a need for further work to establish or to disprove a viral etiologic agent. Similar investigations are suggested for benign calcifying epithelioma of Malherbe (pilomatrixoma of Helwig) which morphologically resembles skin appendageal tumors induced in mice by polyoma virus.

13. Recent virological studies on Kapcsi's sarcoma have been negative but further investigations are justifiable because of the extraordinary variations in incidence reported from Africa (1961 Symposium on Kaposi's Sarcoma, Kampala, Uganda).

14. The possible correlation of certain cancers with common virus infections should be investigated, *e.g.*, whether there is a relationship between herpes simplex infection and carcinoma of the lip.

15. The production of the lesions in man by the Yaba virus has been reported. Investigation of the possible role of this virus in human tumors is recommended.

16. It has been reported that verruca acuminata occasionally progresses to squamous-cell carcinoma. Confirmation of this is desired particularly in populations exposed to high and low risks of squamous-cell carcinoma at the relevant sites.

Pathogenesis:

1. There is need for more information regarding similarities and differences between human skin and skin of animal species used in experiments. We need to know more about regional differences in human skin, and what differences occur in the skin of various human ethnic groups. This applies to morphology, chemistry, vascularity, etc. The use of monkeys for experiments is suggested in countries where they can be maintained.

2. The interaction of carcinogenic agents (chemical, physical, biological) should be studied in animals, including primates and organ cultures with particular reference to the effects on the epidermis, stroma and blood vessels, and at the subcellular level. Attention should be given to terminology of the different agents and their specific action in the neoplastic process.

3. The role of arsenic as carcinogen in man should be further explored and the tissue and blood chemistry should be studied. Experiments on monkeys are recommended.

4. The primary metabolic effect and the role of enhancing factors in xeroderma pigmentosum should be studied on a national and, if possible, international level.

5. More information is needed about the natural history of intraepidermal cancer.

6. More consideration should be given to prophylaxis against ultraviolet cancers by developing protective compounds.

Subcommittee on the Skin as an Assay System for Potential Carcinogens:

1. Carcinogenic response in any animal species raises the question of potential carcinogenicity for man. On the other hand, the short lifespan of the commonly used laboratory animal does not give assurance that negative results in the animal would necessarily yield analogous negative results in the human with protracted exposure. Each agent to be assayed for carcinogenic activity should therefore be tested by several routes of administration in several species and at different dosage. Results of carcinogenic assays should include indication of the total number of tumors induced, their sites, latent period, and the gross and histopathological diagnosis. At least one repetition of each test is advisable.

2. In view of the observed carcinogenic reactivity of human skin to agents assumed to be noncarcinogenic for animals, utilization of human skin, *in vitro*, to study carcinogenesis is suggested.

3. Geographic differences in the occurrence of human skin cancer indicate the need for laboratory identification of specific agents. More precise descriptions of environmental factors associated with human skin cancer will help in the design of laboratory experiments to test their etiologic role.

4. Rapid tests for the assay of mouse skin carcinogens which show promise include the tetrazolium reduction, sebaceous gland suppression, sulfhydryl-disulfide alteration, and newt tail tests. All tests have demonstrated ability to detect some carcinogenic stimuli. Further quantitative studies, at different dose levels of carcinogens, noncarcinogens, and chemical agents, to determine the specificity and sensitivity of the tests indicated are recommended. Several species should be used when possible. The development of other reliable predictive techniques should be encouraged.

5. For the grading of agents to determine relative carcinogenic potency, standardized test procedures should be developed for substances of similar modes of action only, *e.g.*, topical skin carcinogens, radiation. This will necessarily lead to different indices for expressing carcinogenic potency.

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Studies of Development, Function, and Disease

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PREFACE

This Symposium on Studies of Development, Function, and Disease in Organ Cultures comes as a sequel to another, conducted a year earlier, on the subject of Analytic Cell Culture. Both were sponsored by the Tissue Culture Association, and both appear as National Cancer Institute Monographs.

Readers may surmise that the Association took particular care to place these topics in sequence. One can think of many ways in which they supplement one another, or provide opportunities for presentation of opposite views on controversial matters. However, the editor of the present volume has been advised by representatives of the Association that the selection of these two themes, in the order in which they appear, was largely a fortuitous happening. Thus it is on his own that the editor chooses to expound on the wisdom inherent in a random event.

To do this, it is essential first to ask what scientific gain could result from the linking of these unlike, though not unrelated, topics.

In pursuit of an answer, let it be granted that the users of culture methods tend to aggregate into two categories, one composed of cell-culture users and the other of organ-culture users. Spectators may marvel at the similarity of this grouping tendency to the reaggregation phenomenon, which takes place at the cellular level and does not require exercise of cerebral control. This comparison does not suggest that investigators of life processes *in vitro* are a decerebrate lot. It merely emphasizes that investigators, like cells, are wont to join with those who display similar special functions, avidities, and tropisms.

The differences of interest between the two schools can be recognized easily. The school of cell-culture analysts displays an admirable compulsion to achieve the ultimate in simplicity, stability, and uniformity in culture systems. Its disciples strive to exclude all but a single, precisely defined cell type from any given culture or set of replicate cultures. The objectives and methods of this school generally parallel those of microbiologists and include cultivation of dissociated cells, clonal analysis, culture in chemically defined media, quantitative growth studies, somatic cytogenetics, subcellular morphology and biochemistry, and studies of cell-virus interactions.

In contrast, the organ-culture school, or cell society analysts, appear to prefer the more complex to the simpler systems. This view can be supported, at least, if one agrees that a system containing two or more cell types contains more variables than a system containing only one cell type.

The objectives of the organ-culture group are to discover rules that govern development and organization of complex cell societies; to identify natural mechanisms of regulation of growth and function; and to determine how genetic or environmental influences including physical, molecular, and microbial agents produce effects at the cellular and subcellular levels, whence functional changes must further be traced stepwise upward until their effects on a whole organ, several organs, or an organism are determined. The prime needs of this school are methods to maintain morphologic and biochemical homeostasis comparable to that existing in organs and tissues of intact metazoans, including man.

To many, the aspirations of both these schools must appear unrealistic and incapable of fulfillment. In aiming for the ultimate in simplicity, one generally runs the risk of hitting the ultimate in artificiality. By definition, no cell or cell type lives as a hermit or in a strictly isolated cloister within a multicellular higher animal. Nor does any cell or group of cells live long in a protein-free medium within a higher animal. Although it may be claimed justly that there is no substitute for precise laboratory control of culture conditions, at the same time it must be realized that cultures themselves are only substitutes for natural systems, and their fidelity as models is often not only undetermined, but undeterminable.

On the other hand, neither can organ cultures without vascular supplies be looked upon as replications of natural systems. In such cultures the concentration gradients of gasses, nutrient molecules, and excretory or functional products are twisted drastically awry. Organ culturists submit their cultures to conditions more or less befitting protozoans or simple avascular metazoans, while expecting them to perform like cell societies from species at much higher levels of evolutionary development.

Yet, if a homeostatic complexity exactly replicating that of organs *in vivo* could be achieved *in vitro*, there would be little value in the method, because we are already abundantly supplied with animals and organs whose baffling complexity was the *raison d'etre* for most culture research in the first place. The creation of a system with some decrease in complexity, or at least with some alteration in the pattern of complexity without loss of the essential components of the *in vivo* state, is a *sine qua non* of organ-culture analysis as well as of cell-culture analysis.

There is, then, much to be learned from controlled distortion of natural systems. Indeed, it has often been noted that a valuable product of the study of disease is an improved understanding of the normal. Disease can generally be interpreted as a distortion of normal relationships, and it is often this recognizable distortion that first allows investigators to envision the nature of undistorted structure or function. An example is the classic experiment of Metchnikoff, in which the introduction of a rose thorn into the tissues of a starfish larva revealed the origin of phagocytes from fixed mesodermal cells. This discovery was made possible by the addition of a crude artifact to a vastly simplified system lacking the variables of nervous and circulatory components.

In the past, *in vitro* replication of a disease system has involved distortion of the disease just as disease *in vivo* is a distortion of the normal. Herein lie some weaknesses as well as strengths of culture methods. The investigator who uses these methods can move toward greater objectivity and more critical interpretation of his results only when he admits that he is studying distortions or even distortions of distortions.

Many of these thoughts have been expressed by others and with greater clarity. They have led experienced investigators using either cell culture or organ-culture methods to insist upon continuous reference to standards *in vivo* for evaluation of results. Seldom, however, have students of the organ-culture school looked to the cell-culture school for standards of reference, or vice versa. Does this reluctance to rely upon the experience of others show how readily we can detect the flaws in the efforts of an allied group while we retain a blind spot for the flaws in our own work? Are we so preoccupied with one area of interest that we neglect another that could be studied with profit?

Whatever the explanation, it must be confessed that a considerable degree of independence for both schools is quite desirable. One can well imagine the dangers that would beset cell-culture experiments if their interpretation were to rely entirely on organ-culture experiments as a frame of reference, or the converse. If this were to become exclusive practice, much of the resulting thought structure would indeed belong to an unreal world. Surrealism has its appeal in the world of art, but science has no need to nurse a cult of parabiology.

So we grant limits to the extent to which organ-culture studies and cell-culture studies can complement and reinforce each other. But have these limitations been explored and defined? May not some of the more firmly established landmarks in the fields of organ culture and cell culture be used as tentative cross-reference points, without losing sight of the primary reference points provided by living organisms? May not some valuable leads be overlooked if minds of both schools fail to look to each other more frequently than in the past? May some new approaches be developed through a closer approximation of the two areas of interest?

These queries must remain unanswered for the present, but they may suffice to recommend the chronologic apposition of the two Symposia dealing with cell culture and organ culture. They may also give readers an interrogative thread to follow in studying the reports in this volume.

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Session I

Studies of Development in Organ Culture

Chairman: Clifford Grobstein, *Stanford University, Palo Alto, California*

STUDIES ON THE DEVELOPMENT OF EMBRYONIC CARTILAGINOUS LONG-BONE RUDIMENTS *IN VITRO* [1, 2]

J. D. BIGGERS, King Ranch Laboratory of Reproductive Physiology, University of Pennsylvania, Philadelphia, Pennsylvania

SUMMARY

The cartilaginous long-bone rudiments from chick embryos grow in length, and show an increase in wet weight, dry weight, and deoxyribonucleic acid (DNA) content when cultivated on chemically defined media. However, the pattern of response is not the same as when the rudiments are cultivated on natural media. The bones cultivated on chemically defined media elongate more than those cultivated on natural media, but they increase less in wet and dry weight. This organ-culture system has been used to study general problems involved in the design of chemically defined media, such as the determination of concentration-response surfaces to find optimal mixtures. These questions are illustrated by experimental work on the responses to media containing various concentrations of L-glutamine and L-glutamic acid, and by theoretical aspects of investigating the responses on a complex mixture of several amino acids. This organ-culture system has also been used to follow changes in the dry-weight/wet-weight ratio from the time of explantation until the final breakdown of the cultures. The results are discussed with reference to the severe changes induced in tissues by transferring them to a new environment, and the final disruption of organ cultures as the result of breakdown of the intercellular matrices.—NCI Monogr. No. 11: 1–21, 1963.

CARTILAGINOUS EMBRYONIC long bones and other skeletal rudiments have been studied in organ culture for more than 30 years. The first description of the growth and differentiation of cartilaginous rudiments *in vitro* was given by Strangeways and Fell (*1*). They cultivated the limb buds from 3-day chick embryos on a plasma clot, which contained chick-embryo extract, and demonstrated that nodules of cartilage developed in the axial mesoderm. Soon after, Demuth (*2*) and Fell and Robison (*3*) showed that, if cartilaginous primordia of embryonic long bones are explanted, they grow, differentiate, and maintain their characteristic shape.

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[1] Presented at The 13th Annual Meeting of the Tissue Culture Association, Washington, D.C., May 29–31, 1962.

[2] Supported in part by grant C-161-60 from the National Cancer Institute, National Institutes of Health, Public Health Service, and by grant DRG-589A-T from the Damon Runyon Memorial Fund for Cancer Research, Inc.

Since this time many other studies have been made of organ cultures of skeletal rudiments [*see* Fell (*4*) for a detailed review of the literature]. At first these studies were largely concerned with morphological problems, such as the analysis of the development of the budgerigar sternum (*5*), the work being extended later by Chen (*6*) to an analysis of the development of the mouse sternum. Although Fell and Robison (*3*) showed in 1929 that organ cultures of embryonic chick femora could be profitably studied by biochemical techniques, the physiology and biochemical properties of explanted cartilaginous rudiments have been taken up seriously only during the last 15 years. Now a great many studies have been done, such as those of Fell and her colleagues on the action of vitamin A (*7–9*), Gaillard on the action of the parathyroid hormone (*10, 11*), and Wolff and his colleagues on various methods of organ culture, which included pioneer studies on the growth of embryonic long bones on chemically defined media (*12, 13*).

Some tissues are easier to set up as organ cultures than others; hence, investigators who have wanted to utilize the method on a particularly difficult tissue have often been frustrated. Embryonic long-bone rudiments, however, are one of the easiest tissues to explant and are, therefore, model structures for the quantitative study of organ-culture systems. Quantitative studies of the responses of chick and mammalian long-bone rudiments on chemically defined media have led me to consider problems and phenomena likely to occur generally in organ-culture systems. Since their understanding is essential for the proper interpretation of experiments and may help in studies on more refractory tissues, I wish to concentrate on these problems in this paper. First the responses of embryonic long bones on chemically defined media will be briefly reviewed, which will include a description of the methods used in my laboratory. Then the problems of the study of the responses of these rudiments quantitatively will be discussed, with particular reference to the comparison of growth on chemically defined and natural media and the requirements for L-glutamine. Finally, problems associated with the movement of water in and out of the explants will be discussed.

MATERIALS AND METHODS

Explants.—Tibiotarsi from 7-day chick embryos, tibiae from 17-day rat embryos, and tibiae from 15-day mouse embryos were used. All 3 types of rudiments are miniature cartilaginous templates of the adult bones produced by endochondral ossification. Each rudiment consists of a rod of cartilage surrounded by a perichondrial sheath. In the diaphyseal region a thin lamina of periosteal bone lies between the cartilage and perichondrium. Growth and differentiation of these structures occur by 3 basic cellular processes: cell division, secretion of intercellular matrix, and hypertrophy.

Culture technique.—The rudiments were cultivated at 37° C, with the use of Chen's method for liquid media (*14*), either in humid air or 5 percent carbon dioxide in air.

Chemically defined media.—Two types of chemically defined media were used, one relatively complex and the other relatively simple. The complex medium was No. 858 (*15*) and a minor variant of it denoted BLI (*16*). The simple medium has been designed recently in my laboratory and is denoted BGJ (*17*).

The composition of medium BGJ is shown in table 1. It contains 12 amino acids and L-glutamine, which we consider essential nutrients for the growth of embryonic chick tibiotarsi and embryonic rat tibiae. The reasons for these conclusions, which do not agree with the earlier findings of Wolff and his colleagues, summarized by Kilny (*13*), are given elsewhere (*17–20*). So far we have made no critical studies to determine whether the remaining components of medium BGJ are essential.

TABLE 1.—Chemically defined medium BGJ*b*

	mg per 100 ml	mM		mg per 100 ml
L-Lysine hydrochloride	24. 0	1. 31	Nicotinamide	2. 0
L-Histidine hyrdochloride H_2O	15. 0	0. 72	Thiamine hydrochloride	0. 4
L-Arginine hydrochloride	7. 5	0. 36	Calcium pantothenate	0. 02
L-Threonine	7. 5	0. 63	Riboflavine	0. 02
L-Valine	6. 5	0. 56	Pyridoxal phosphate	0. 02
L-Leucine	5. 0	0. 38	Folic acid	0. 02
L-Isoleucine	3. 0	0. 23	Biotin	0. 02
L-Methionine	5. 0	0. 34	*p*-Aminobenzoic acid	0. 2
L-Phenylalanine	5. 0	0. 30	α-Tocopherol phosphate (sodium salt)	0. 1
L-Tryptophan	4. 0	0. 20		
L-Tyrosine	4. 0	0. 22	Choline chloride	5. 0
L-Cysteine hydrochloride H_2O	9. 0	0. 51	*m*-Inositol	0. 02
L-Glutamine	20. 0	1. 37	Vitamin B_{12}	0. 004
Calcium lactate	55. 5		Glucose	500. 0
Potassium chloride	53. 0		Streptomycin	5. 0
Potassium dihydrogen phosphate	16. 0		Penicillin	10,000 units
Sodium chloride	800. 0			
Magnesium sulfate (7 H_2O)	20. 0			
Sodium bicarbonate	350. 0			

EXPERIMENTAL DESIGNS

The study of concentration-response lines and concentration-response surfaces (*21*) is essential for the quantitative study of the responses of explanted tissues on different media. Since these studies can require an often expensive, tedious amount of work, utilization of the most efficient experimental designs that are available is important [*see* Cox (*22*) for a nonmathematical introduction to the theory of the design of experiments]. Our work on embryonic long-bone rudiments has been facilitated immeasurably by exploitation of the uniformity between the paired rudiments from each embryo. Two main types of design have been used:

1) Split-plot designs, particularly useful for the comparison of 2 growth curves or 2 dose-response lines (*23*).

2) Balanced incomplete block designs with 2 plots per block. These designs are particularly useful for the efficient determination of dose-response lines (*24*) and for experiments in which combinations of 2 or more factors are arranged in a factorial scheme (*18*, *19*). Further information on the use of these designs is given elsewhere (*20*, *23*).

A corollary to the use of these designs is that it must always be possible to make determinations on individual rudiments. It was for this reason that considerable attention was given to the methods for making dry-weight and wet-weight determinations on single bones with the use of an electric microbalance (*23*) and for deoxyribonucleic acid (DNA) determinations on single bones (*20*).

RESPONSES OF EMBRYONIC LONG BONES ON CHEMICALLY DEFINED MEDIA (*17*, *25*)

Seven-day embryonic chick tibiotarsi were cultivated for 8 days on medium BGJ, with the medium renewed on alternate days. Initially and every 2d day, 3 rudiments were removed, and their lengths, wet weights, and DNA contents determined.[3] The results are shown in text-figure 1. The rudiments increased in length from 3.3 to 8.9 mm on the 8th day. The slight drop in length between the 6th and 8th days is not significant. The rudiments also increased continuously in wet weight from 1.0 to 6.8 mg. The rudiments increased in DNA content from 1.05 μg initially to 2.90 μg on the 6th day; thereafter the DNA decreased to 2.15 μg on the 8th day. This rather large fall between the 6th and 8th days, however, appears to be spurious, since subsequent experiments have shown that the DNA content tends to remain constant after the 6th day *in vitro* (*25*). These rudiments also retained their shape, apart from varying degrees of bending of the diaphysis. Other studies, with the use of histological and autoradiographical methods after exposure of the rudiments to tritiated thymidine, have shown that all 3 basic cellular processes that determine the shape of the rudiment—cell division, secretion of matrix, and hypertrophy—occur in rudiments cultivated on medium BGJ. There is no doubt, therefore, that 7-day embryonic chick tibiotarsi grow when cultivated on medium BGJ. Other experiments have shown that tibiae from 17-day rat embryos also grow on this medium. As yet, however, no differences have been found between the growth that occurs on medium BGJ and the more complex chemically defined media of the medium 858 family.

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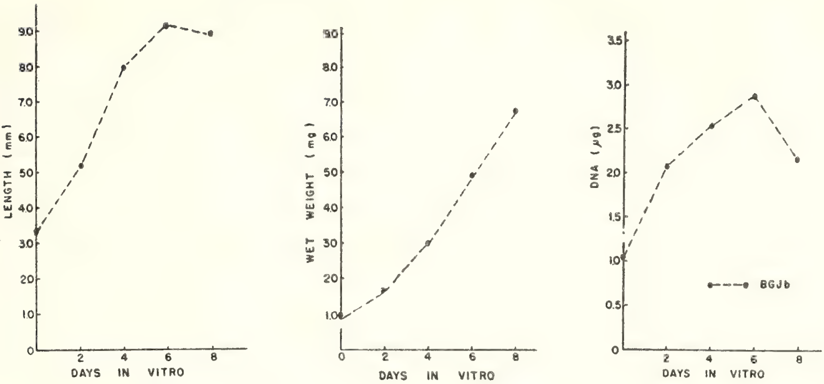
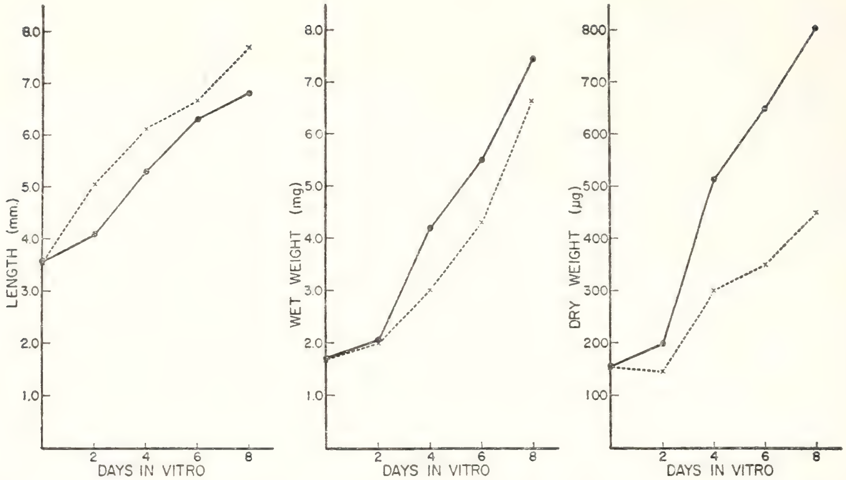
[3] These results are part of a larger split-plot experiment in which the responses on medium BGJ and a natural medium were compared (*25*). Since we were concerned primarily with differences in the responses on the 2 media, the day-to-day responses shown in text-figure 1 are relatively ill-determined, being subject to the large between embryo (whole-plot) variation.



TEXT-FIGURE 1.—The changes in length, wet weight, and deoxyribonucleic acid (DNA) content of 7-day embryonic chick tibiotarsi cultivated on medium BGJ. Each mean is based on observations of 3 tibiotarsi from different embryos (*see* footnote 3).

COMPARISON OF RESPONSES OF EMBRYONIC LONG BONES ON CHEMICALLY DEFINED AND NATURAL MEDIA (*26–28*)

The changes in length, wet weight, and dry weight of 7-day embryonic chick tibiotarsi were compared after cultivation on medium BLI and chicken serum supplemented with chick-embryo extract. Medium BLI contained 4.0 mg per ml glucose, and the same amount of glucose was added to the natural medium. This procedure eliminates the possibility of observed differences being caused by uncontrolled variations in the concentration of glucose in the 2 media, by ensuring that observations are made on the asymptotic region of the glucose concentration-response curve in both cases (*24*). The comparison of the 2 media was made with the use of a split-plot experimental design, and the results and their analysis are described in detail elsewhere (*26*). The results are summarized in text-figure 2. The bones cultivated on medium BLI elongated over the 8-day period from 3.57 to 7.64 mm, while those cultivated on the natural medium elongated from 3.57 to 6.81 mm. The differences between the 2 growth curves are highly significant. The same bones increased in wet weight from 1.36 to 6.41 mg on medium BLI and 1.36 to 7.47 mg on the natural medium. They also increased in dry weight from 181 to 454 μg and 181 to 807 μg on medium BLI and the natural medium, respectively. These differences between the increases in wet and dry weight on the 2 media are highly significant. Thus this experiment shows that, although the rudiments cultivated on medium BLI elongated more than those cultivated on the natural medium, they grew significantly less in wet and dry weight. These observations have been fully confirmed by experiments that compare the responses in length and wet and dry weight of 17-day embryonic rat tibiae on medium BGJ and the corresponding homologous natural media (*26–28*).



Text-figure 2.—The changes in length, wet weight, and dry weight of 7-day embryonic chick tibiotarsi cultivated on medium BLI and chicken serum supplemented with chick-embryo extract. The pair of means shown for each day is precisely determined with respect to each other because the comparison of the media was made in a split-plot experimental design; each mean is based on 3 observations.

CONCENTRATION-RESPONSE SURFACES

An organ-culture system may be considered in simple abstract terms as comprising 2 phases: the explant and the medium. The phases are not independent since they interact with each other from the moment the organ culture is set up.

Explant

The explant consists of an organized collection of cells, not necessarily of one type, held together by an intercellular matrix. An essential difference between an organ culture and a cell culture is that only in the organ culture is the living structure organized, in the sense that the constituent cells form a cohesive structure having an identity of its own, in which each cell influences the other cells via the intercellular matrix. Furthermore, this matrix, chemically complex, is an integral part of the culture and has to be produced and maintained by the constituent cells.[4] An individual explant, such as a cartilaginous long-bone rudiment, therefore, has *macroscopic* properties—length, total wet weight, total hydroxyproline content, total DNA content, etc.—and *microscopic* properties, such as the rates of synthesis of collagen and chondroitin sulfate by each cell within the explant and the concentration of Na^+ and K^+ near each cell membrane.

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[4] It seems that some organotypic cultures, such as are produced in Rose chambers (*29*), are intermediate between cell and organ cultures in terms of the relative importance of cells and matrix, and may, therefore, have different properties.

Medium

The medium may or may not be chemically defined. A chemically defined medium means that the chemical composition of the medium is rigorously known, which includes exact information on the composition of the gas phase to which the medium is exposed. The medium is not chemically defined if biological fluids or partially purified biological products, such as dialyzed serum, are included.

Relation Between Explant and Medium

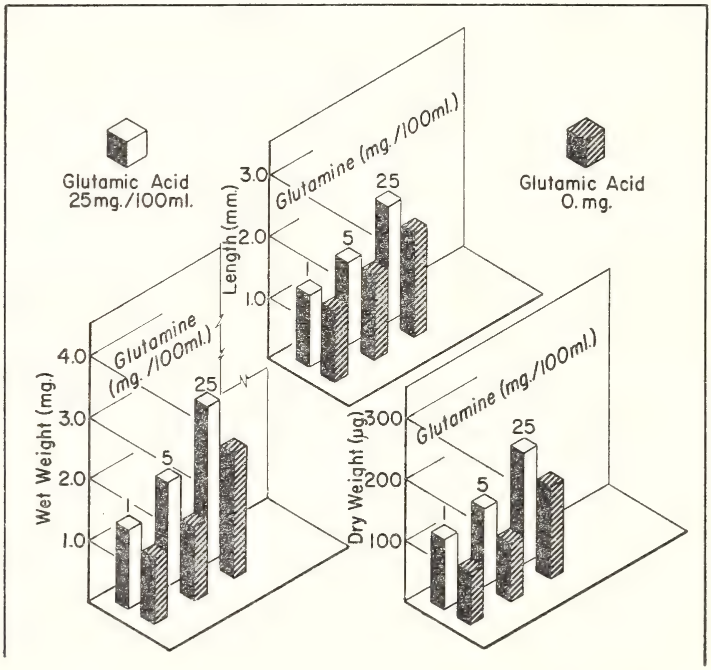
The following discussion assumes that the medium is chemically defined and consists of n chemical constituents. The basic ideas also apply to situations with media of unknown, or partially known, composition, the difference being that the medium contains $(n + x)$ components, x of them unknown $(x = 1, 2, \ldots)$. A fundamental fact is that the composition of a chemically defined medium is known only at the time of explantation, since as soon as a tissue is placed in contact with a medium, exchanges take place between them. There is ample experimental evidence for this fact from countless studies with short-term experiments on tissue slices. In organ cultures of embryonic chick long bones, there are several studies showing large changes in the composition of chemically defined media and the explants after a period of cultivation (*21*, *30–34*).

In 1957, Biggers, Rinaldini, and Webb (*21*) discussed theoretically the responses of tissue cultures on media of varying composition. It was pointed out that a particular response could be regarded as a dependent variable subject to variations in the concentration of each of i compounds in the medium $(i = 1, 2, \ldots, n)$. If we visualize this geometrically, the locus of all possible responses will form a concentration-response surface in i-dimensional space. Thus, in general, the practical experimental problem is to explore and map this surface.

This concept will be illustrated in concrete terms by 2 problems: (*a*) the response of embryonic chick tibiotarsi to various combinations of L-glutamine and L-glutamic acid and (*b*) the determination of the amino acid requirements of embryonic long-bone rudiments.

In initial studies on the growth of avian and mammalian long-bone rudiments on medium BGJ (*17*), it was shown that L-glutamine was essential for growth. However, other studies have shown that L-glutamic acid is synthesized from glucose by embryonic chick tibiotarsi (*32*) and that L-glutamine can be synthesized from L-glutamic acid by embryonic chick heart tissue, the reaction being catalyzed by the enzyme glutamine synthetase (*35*). Thus it is possible that some L-glutamine is produced from glucose by embryonic bones cultivated on medium BGJ. The possibility of interaction of effects between 3 concentrations of L-glutamine and the presence and absence of L-glutamic acid in the medium was therefore investigated with a 2 × 3 factorial experimental design.

Seven-day embryonic chick tibiotarsi were cultivated for 6 days on medium BGJ, which contained 1, 5, and 25 mg per 100 ml L-glutamine in the presence of 0 and 25 mg per 100 ml L-glutamic acid. At the end of the experiment the lengths, wet weights, and dry weights of the rudiments were determined. The results are illustrated in text-figure 3. Since the full details and statistical analyses of this experiment have been published elsewhere (*18, 19*), only the essential features of the experiment will be discussed. Although the responses in length, wet weight, and dry weight of the rudiments are highly dependent on the concentration of L-glutamine in the medium, the concentration of L-glutamic acid has no effect on the length of the rudiments but has a significant effect on the wet and dry weight. In this particular experiment there was no suggestion of interaction of effects in which the concentration of L-glutamine controlled whether or not the presence of L-glutamic acid had an effect. A very important conclusion from this experiment is that the nature of a concentration-response surface can depend on the particular response studied.[5] As a result of the demonstration of this difference, however, it was concluded from the studies on length that embryonic chick tibiotarsi do not synthesize significant amounts of L-glutamine from L-glutamic acid; nevertheless concomitant studies on wet and dry weight showed that L-glutamic acid does increase



TEXT-FIGURE 3.—The results of a 2 × 3 factorial experiment, showing the effect of 1, 5, and 25 mg percent L-glutamine and the presence of 0 and 25 mg percent L-glutamic acid on the length, wet weight, and dry weight of 7-day embryonic chick tibiotarsi cultivated for 6 days on medium BGJ.

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[5] A trivariate example of this fact has been described before in this paper, where it was shown that, on any given day after explantation, embryonic chick tibiotarsi elongated more on medium BLI than on a natural medium, but increased less in wet and dry weight on the 2 media, respectively.

the growth of these rudiments even in the presence of maximal concentrations of L-glutamine.

The problem of the determination of the amino acids which are essential for the growth of embryonic chick tibiotarsi and embryonic rat tibiae has been extensively studied by Biggers and Heyner (*20*). The final list of 12 amino acids, incorporated in medium BGJ, was arrived at by the investigation of the effect of complete deficiencies examined one at a time and from studies on the metabolism of glucose and amino acids with the use of isotopically labeled compounds (*32*). The concentrations of the amino acids in medium BGJ are still arbitrary and are largely those adopted by earlier workers for cell culture, based on analyses of plasma proteins. It is likely that the concentrations of the 12 amino acids required to give some optimal response will not be independent of each other; in other words, the required concentration of a particular amino acid will depend on the concentrations of at least some of the other 11 amino acids. Unfortunately, the study of the possibilities requires the examination of a 12-dimensional concentration-response surface with the use of a w^{12} factorial experiment, where w is the number of different concentrations of each amino acid employed. Obviously the larger the w the better will we estimate the nature of the concentration-response surface. However, even if we attempt to estimate the surface crudely by using only a low and a high concentration of each amino acid ($w = 2$), 4096 media would still have to be prepared, a completely impractical task. Nevertheless some progress can be made with the fractional factorial experimental designs (*36*) in which only a fraction of the total number of media is required. Furthermore, such experiments can also be readily analyzed statistically, since multivariate analyses of variance programs are now available for high-speed digital computors (*37*).

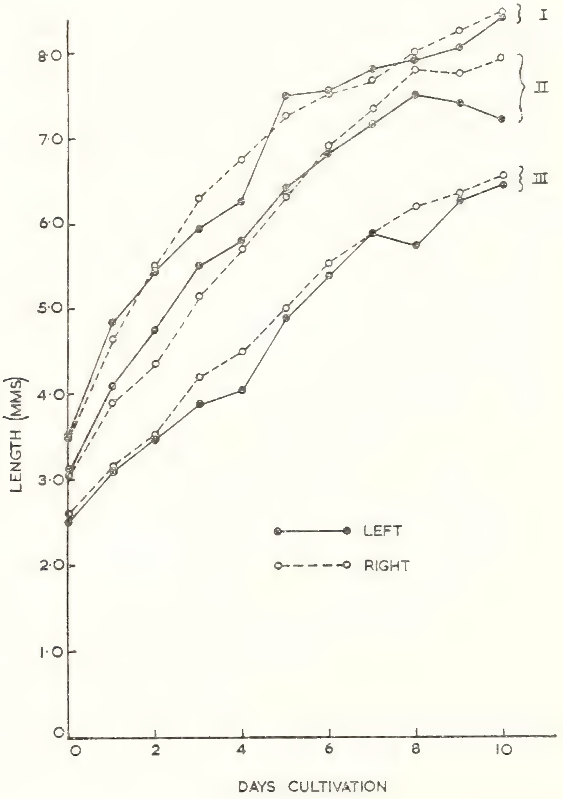
The exploration of complex concentration-response surfaces has been developed to a high degree in the field of chemistry. Further details on problems connected with setting up suitable mathematical models and experimental designs in this type of work are given by Box and Wilson (*38*) and Box (*39*).

STABILITY OF ORGAN CULTURES OF EMBRYONIC LONG-BONE RUDIMENTS

It is well known that the genotype of an embryo influences the development of portions of it *in vitro*. Thus in a recent study, Abbott and Kieny (*40*) showed that the tibiotarsi from mutant chick embryos (Diplopodia) grow less than do normal tibiotarsi after explantation, just as they do *in vivo*. It is also realized that, when embryonic long-bone rudiments are explanted, they are already differentiated in the sense that a tibiotarsal rudiment from an embryo will grow and differentiate into a larger rudiment having the shape of a tibiotarsus, and a radial rudiment from the same embryo will grow simultaneously into a larger rudiment having the shape of a radius. Furthermore, during the early stages of development 2 long-bone rudiments may differentiate so that they react differently to external agents. An excellent example of this phenomenon was demonstrated by Fell and Mellanby *(41)* who showed that L-thyroxine inhibits the elongation of embryonic chick tibiae and femora *in vitro* and stimulates the elongation of the radius.

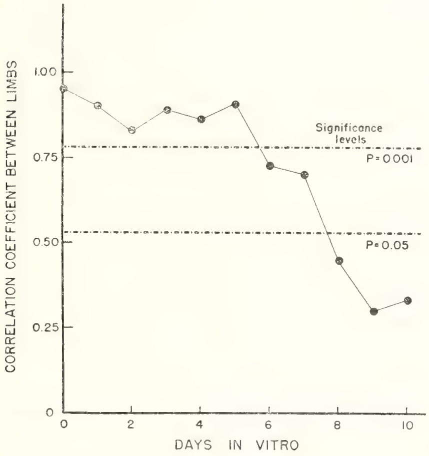
Another example, which I wish to consider, differs somewhat in nature from those just cited.

Text-figure 4 shows the growth in length of the paired tibiotarsi from three 7-day chick embryos, cultivated in separate dishes on medium BLI. All the rudiments elongated by about 100 percent, and the paired bones, having the same genotype, tended to respond similarly. The results shown in text-figure 4, however, were chosen, for the sake of clarity, from an experiment in which the growth of the paired tibiotarsi from 12 embryos was studied. The degree of association between the paired rudiments on any given day can be estimated quantitatively by the coefficient of correlation. These coefficients are shown in text-figure 5. Initially and for the first 5 days after explantation, the correlation coefficients are large,

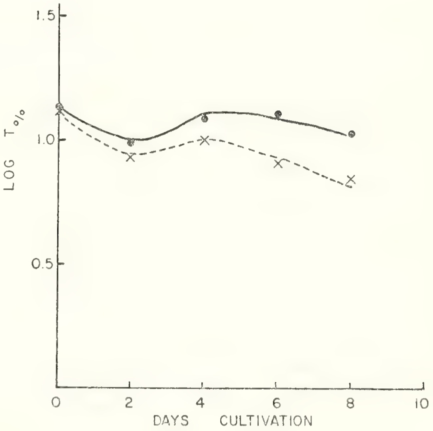


Text-figure 4.—The length of the left and right tibiotarsi from three 7-day chick embryos on each of 10 days after explantation on medium BLI containing 4.0 mg glucose per ml.

highly significant, positive values. After the 5th day, however, the degree of correlation rapidly falls. Nevertheless, the rudiments continue to grow during the period of declining correlation between the responses of the paired bones. A change such as this indicates the onset of instability in the system in which the genetic and permanent environmental factors, which have determined the properties of the rudiment before explantation, become overruled by new environmental influences arising *in vitro*. The fall in correlation may not mean the cells of the explant are dying, but reflects a loss of organization.



Text-figure 5.—The coefficients of correlation between the left and right tibiotarsi from twelve 7-day chick embryos on each of the 10 days after explantation on medium BLI containing 4.0 mg glucose per ml.



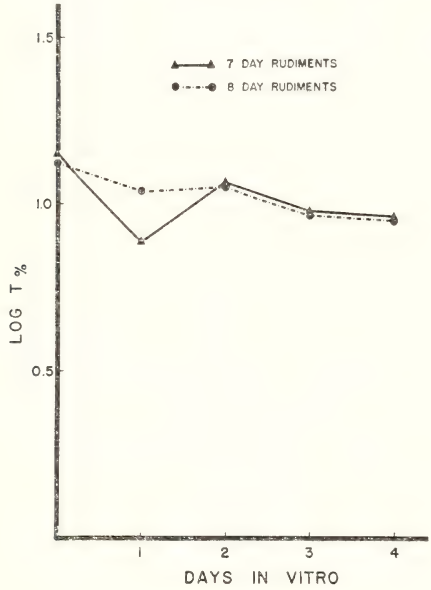
Text-figure 6.—The log dry-weight/wet-weight ratio (log $T_{\%}$) of tibiotarsi from 7-day chick embryos cultivated on medium BLI (x__ __ __x) and chicken serum supplemented with embryo extract (●———●).

DRY-WEIGHT/WET-WEIGHT RATIO

A particularly useful guide to the condition of cells is their dry-weight/wet-weight ratio, for, under conditions of stress and as a prelude to early degeneration, such as cloudy swelling, they often become markedly hydrated. By using a microbalance, we routinely measured the dry weight and the wet weight of individual long-bone rudiments and calculated the dry-weight/wet-weight ratio (*23*). The results shown in text-figure 6 are the dry-weight/wet-weight ratios calculated from the experiment previously shown in text-figure 2. These results compare the changes in the ratio of embryonic chick tibiotarsi cultivated on chicken serum supplemented with embryo extract and on the chemically defined medium BLI. The changes in the bones follow the same pattern on both media. First, the ratio declines, then it increases to a level equal to or somewhat less than the initial level, and after a brief period of stability the ratio slowly declines. For convenience these phases will be referred to as: 1) the initial "shock" phase, 2) the middle "stable" phase, and 3) the final phase of slow hydration. The rest of this review will be concerned with the first and third of these phases.

Initial "Shock" Phase

The changes in the dry-weight/wet-weight ratio have been examined in more detail with the tibiotarsi from 7- and 8-day chick embryos cultivated on medium BGJ. The results are shown in text-figure 7. The graphs demonstrate that the dry-weight/wet-weight ratio of the 7-day rudiments falls rapidly during the first 24 hours and then recovers partially during the second 24 hours. Similar, but less severe, changes occur in the 8-day rudiments; nevertheless, the changes are highly significant (*42*).



Text-figure 7.—The log dry-weight/wet-weight ratio (log $T_{\%}$) of tibiotarsi from 7- and 8-day chick embryos cultivated on medium BGJ.

The fact that large initial changes occur in the dry-weight/wet-weight ratio of explants raises the problem of the biochemical changes occurring immediately after explantation. These should be studied, since, by understanding these phenomena, we might gain answers to such problems as: 1) why it is difficult to get small embryonic chick-bone rudiments, such as 5-day tibiotarsi, to grow on chemically defined media and 2) why it is more difficult to get mammalian embryonic bones rather than avian bones to grow, for, once the deleterious changes are understood, there is always the possibility of modifying the conditions to minimize them.

Unfortunately this problem has received no study so far. However a number of papers on short-term studies *in vitro* indicates roughly the type of changes that may occur. For example, Judah (*43*) reported a considerable loss of malic dehydrogenase from liver slices during the first 30 minutes in physiological saline. Kidney and liver slices lose potassium and take up water and sodium, if placed in cold sodium chloride solution, and these changes are reversed when the slices are transferred to warm oxygenated Ringer's solution (*44, 45*). Christensen and his colleagues (*46*) have shown that mammalian cells can rapidly concentrate amino acids, which remain free in the cells and exert full osmotic activity. The presence of raised levels of free amino acids within the cells, in turn, influences the distribution of ions. As implied by Waymouth (*47*), it seems likely that these changes are pronounced when fragments of tissues are explanted, particularly on chemically defined media containing high levels of free amino acids, and may account for the rapid hydration of embryonic chick bones when they are explanted.

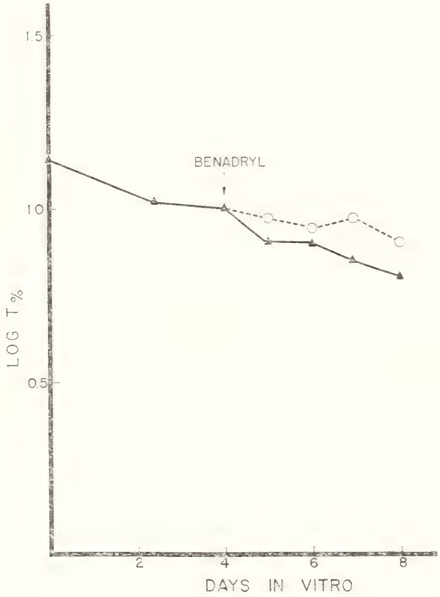
It is also known that many explanted tissues recover from the disturbances arising at the time of explantation. Again, we know nothing of the biochemical aspects of these phenomena in organ cultures. An interesting clue is possibly given by the observations of Broome and Opie (*48*) and Opie (*49*) which suggest that when the amino acids accumulating in liver are oxidized, the urea, which is formed rapidly, leaves the cell and so lowers its internal osmotic pressure. In other tissues that do not possess the enzymes necessary for the production of urea, other mechanisms presumably operate to reduce the level of free amino acids in the cells.

However, in the cultures of embryonic chick bones, a "stable" phase develops, which implies a considerable degree of adaptation to the new conditions created each time the medium is renewed.

Final Phase of Slow Hydration

A characteristic feature of all organ cultures of embryonic bones, which are slowly undergoing disruption, is their hydropic appearance. In our work with chemically defined media, this condition can be detected visually on the 7th to 8th day after explantation. However the studies on the dry-weight/wet-weight ratio show that the hydration commences on the 5th day. This hydration of the embryonic cartilaginous rudiments represents the earliest stage in the breakdown of the intercellular matrix. Since the disruption of intercellular matrices is a common phenomenon in many organ-culture systems, detailed examination of this process is of general interest.

The matrix of embryonic chick long bones consists of collagen fibers bound together by a complex mixture of mucopolysaccharides. Many small molecules, including water, are bound by this complex of macromolecules. However, there is a fixed amount of water normally present, for, if the collagen fibers are disrupted by the incorporation of a lathyrogenic agent in the medium, the matrix takes up more water. This phenomenon is illustrated by the data in table 2, showing the action of various concentrations of methyleneaminoacetonitrile (MAAN) on embryonic chick tibiotarsi (*50*). The rudiments subjected to 10^{-3} M MAAN, for example, grew in length, wet weight, and dry weight. However, they became considerably wetter than the controls since they increased more in wet weight and less in dry weight. Presumably the lower dry weight is caused by loss of matrix from the explant into the medium, and the higher wet weight is due to the binding of more water by the disrupted matrix. It is important for one to realize, however, that hydration of the rudiments occurs only when living cells are present. Thus table 2 shows that bones exposed to 10^{-2} M MAAN have the same dry-weight/wet-weight ratio as the controls. This high concentration of MAAN in fact rapidly kills the rudiments. A similar effect has also been seen in studies on the action of Benadryl on embryonic chick tibiotarsi (*42*). If high, lethal concentrations of this drug are added to the medium, the explants do not become hydrated. The action of the nitrile, therefore, is not through a purely chemical action on the collagen but one also on the cells, perhaps by causing a leakage of enzymes that break down the mucopolysaccharides. It is possible that the breakdown of intercellular matrix under other conditions, such as continued exposure to chemically defined media, is produced by a similar process.



TEXT-FIGURE 8.—Effect of Benadryl (10^{-4} M) on the dry-weight/wet-weight ratio (log *T%*) when added on the 4th day of cultivation *in vitro*.

Recently Biggers and Gwatkin (*42*) studied the action of Benadryl on the hydration of embryonic chick tibiotarsi which occurs after the 5th day on medium BGJ. This substance can stabilize the membranes of cells and their organelles under adverse conditions (*51*). The results, shown in text-figure 8, demonstrate that Benadryl partially inhibits the hydration. This finding lends support to the view that the primary damage involves the cells, which are then unable to maintain the matrix between them. The finding also suggests that other agents, which include hormones and drugs, may be more effective than Benadryl in controlling the effects of mild cellular damage.

TABLE 2.—Effect of methyleneaminoacetonitrile (MAAN) on the responses of 7-day embryonic chick tibiotarsi cultivated on medium BGJ

Concentration MAAN (M)	Length (mm)	Wet weight (mg)	Dry weight (μg)	Dry-weight/wet-weight ratio (%)
0	7. 2	4. 58	437	9. 5
10^{-4}	7. 8	5. 14	452	8. 8
5×10^{-4}	7. 4	5. 12	422	8. 2
10^{-3}	7. 5	5. 05	383	7. 6
5×10^{-3}	3. 7	1. 88	162	8. 6
10^{-2}	3. 2	1. 77	169	9. 5

CONCLUSION

There are several reasons for the increasing interest in the technique of organ culture. Students of development, growth, and differentiation hope that the technique may help to bridge the gap between work on cell cultures and work on entire organisms. More and more students of physiology are finding the technique useful in studies of function, and pathologists are finding it helpful in the study of disease processes. It is essential, therefore, that organ culture is regarded only as a technique which can yield results helping us to understand processes in intact individuals. It is obviously absurd to believe that we can reproduce *in vitro* exactly the conditions *in vivo*, for the very act of isolating a piece of tissue from an intact individual must create abnormal conditions. In this paper, therefore, emphasis has been put on the fact that organ cultures are systems involving the interaction of explant and medium on each other. Phenomena, which occur in intact organisms, will occur and can be usefully studied in these systems, but the results will also be modified by other effects peculiar to the organ-culture conditions. Examples of the latter are the initial shock phase and the final slow disorganization, which were discussed in the final part of this paper. Unless the balance between these phenomena is understood, errors in the interpretation of experiments with organ cultures may easily be made.

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DISCUSSION

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The intrinsic properties of a bone rudiment—whether it is meant to be a radius or a tibia or any other element—may be of vital importance in the determination of its response *in vitro*. Dr. Biggers has provided us with ample evidence from the literature and his own work to prove this.

The second factor Dr. Biggers points to, which was reported by Fell and Mellanby (*1*) and quite recently in detail by Lawson (*2*), concerns the size and the developmental stage of the rudiments at the beginning of the experiment.

To these two factors that determine the response *in vitro*, I should like to add a third one. From experiments with long-bone rudiments of human origin, we believe we have evidence that the age of the donor embryo as such, *i.e.*, comparatively regardless of type, size, and stage of the explant, may be an additional factor in the determination of the behavior of these embryonic bones during the cultivation period.

The experiments to which I refer deal with the influence of epithelial tissues on explants of bone rudiments of hand and foot of human embryos (*3*). The rudiments were cultivated for periods varying from 10 to 28 days on a natural medium with the use of the watchglass technique. Under the given circumstances an epithelial encapsulation of each explant, either with epidermis or with any other epithelial tissue having a sufficient proliferative capacity, appeared to be essential for a normal development *in vitro*. Figure 1 illustrates this phenomenon. Though in the nonencapsulated explants mitotic activity and matrix formation are intensive all over the rudiment, further differentiation in an organotypic way completely fails. As a consequence, after some time the rudiment is hardly recognizable as an embryonic bone. In the encapsulated rudiments, on the other hand, mitotic activity is mainly restricted to a zone of proliferating cells and there is a marked hypertrophy of cells in the diaphysis. The characteristic shape of the element is retained, and we could speak of it as a comparatively normal organotypic development.

The epithelial dependency could not only be demonstrated in phalanges of different types and sizes but also in the larger and more differentiated metacarpal and metatarsal bone rudiments.

The phenomenon invariably appeared if the rudiments were taken from embryos older than 9 weeks. However, when younger embryos were introduced into the experiments, suddenly the presence or absence of an epithelial encapsulation seemed to be of no importance for the development of the bone rudiments *in vitro*. Figure 2 demonstrates that in that case normal development may proceed in the nonencapsulated explant. This photograph of a metacarpal bone rudiment at the same time illustrates our experience—that it is not necessarily more difficult to get mammalian embryonic bones to grow *in vitro* than avian ones. As a matter of fact the specific growth rate, which in the embryonic chick is rather high, is accounted for as far as absolute values are concerned, but relatively speaking the mammalian rudiments often really do not lag. In the explant of figure 2 the rudiment grew even better than it would have done *in vivo*. We have no hydration values at our disposal, but the metachromatic properties of the cartilage matrix remained intact even after 4 weeks of cultivation. The histological picture shows no sign of a cloudy swelling or any other indication of a beginning degeneration of the explants.

Phalangeal rudiments of the same size and developmental stage as the young metacarpal bones, but derived from older embryos (more than 9 weeks old), definitely do require the epithelial encapsulation.

Phalanges from embryos younger than 9 weeks behave in just the same way as the metacarpal bone rudiment of figure 2 and do not seem to need any epithelial covering.

Type, size, and stage thus being eliminated, it is evident that in our experiments the age of the donor embryo is accountable for the difference in behavior of the explants.

With regard to various responses of bone rudiments *in vitro*, the three factors mentioned will surely intermingle. For the investigator in the field of organ culture this implies that, besides a careful interpretation of the results, a thorough knowledge of his material is indispensable.

There is an additional point on which I should be glad to hear Dr. Biggers' opinion. On various chemically defined media, bone rudiments elongate more but increase less in weight than on a natural medium; this seems to be exactly the same difference we found in the group of older human embryos, between the rudiments with and without an epithelial capsule. It would be interesting to know if this similarity could be based on the same histological details. I wonder if it is Dr. Biggers' impression that on BGJ medium the mitotic activity in the small cell cartilage of the epiphyseal region is depressed, whereas the zone of proliferating cells is stimulated, and if anything could be observed of an increased hypertrophy.

If so, we may perhaps speculate along the following lines: In the long and slender bone rudiments, described by Dr. Biggers, organotypic developmental processes seem to be excessively promoted, whereas our bulky nonencapsulated human bone rudiments apparently are unable to hold their organ character. If in either case the medium is deficient, it follows that the epithelial barrier effectively provides the explants in our experiments with a chemically defined medium on which the golden means in organotypic development can be obtained. Unfortunately, however, while Dr. Biggers kindly informed us of the ingredients of his medium, the epithelium has as yet succeeded in keeping its secret.

Comments by Dr. Biggers in Reply to Dr. Zaaijer's Question

I was very interested to hear that Dr. Zaaijer has found differences in the responses of cultures of human long bones with and without epithelial capsules, and that the direction of the differences depended on the response being measured. As far as we can tell, the excessive elongation of embryonic chick tibiotarsi, cultivated on medium BGJ, is caused partly by a burst of mitotic activity in the epiphyses within the first 2 days after explantation, and partly by the zone of hypertrophic cells extending more than usual into the zone of flattened cells. Dr. Susan Heyner and I have shown, in unpublished experiments, that the incorporation of only 10 percent chicken serum in medium BGJ will prevent this excessive elongation. Thus there must be substances present in serum, and presumably in the epithelial capsule in Dr. Zaaijer's experiments, which control and integrate the shapes of the growing rudiments.

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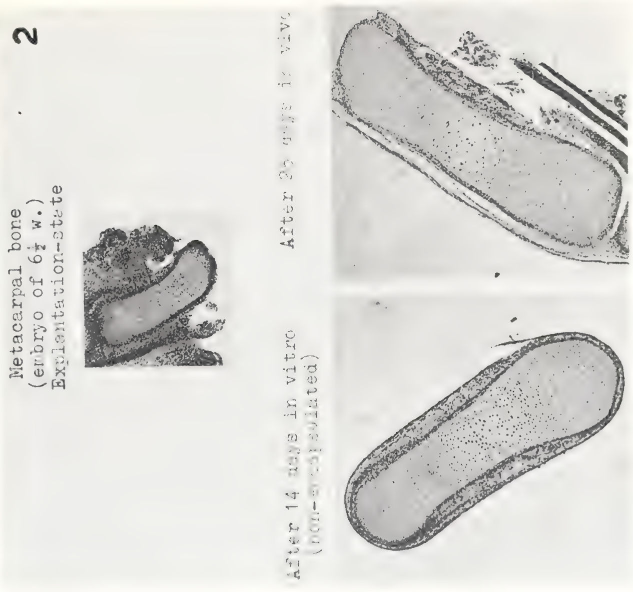
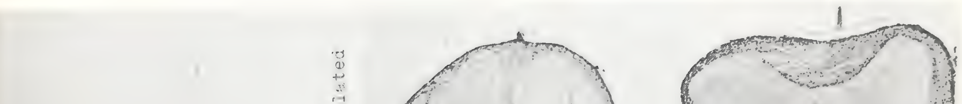
PLATES

PLATE 1

FIGURE 1.—Encapsulated and nonencapsulated explants of proximal phalanx after cultivation for 18 days. Nonencapsulated explants show mitotic activity but fail to differentiate in an organotypic way. Mitotic activity of encapsulated explants is restricted and there is a comparatively normal organotypic development.

FIGURE 2.—*Upper center:* metacarpal bone rudiment at time of explantation. *Lower left:* equivalent rudiment after 14 days in organ culture. *Lower right:* metacarpal bone of a 10-week-old embryo (not cultured).


2
Metacarpal bone
(embryo of 6½ w.)
Explantation-state
After 14 days in vitro
(non-encapsulated)
After 26 days in vivo


1
Proximal phalanx
(embryo of 10½ w.)
Explantation-state
After 18 days of cultivation
Encapsulated
Non-encapsulated

ZAAIJER

DEVELOPMENTAL STUDIES OF MOUSE THYMUS AND SPLEEN[1, 2]

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SUMMARY

Experiments were designed to examine critically several aspects of the hypothesis that the thymus represents the major primordium of the mammalian immunological system. When embryonic spleen rudiments were grown *in vitro*, they failed to develop further. When transplanted into the anterior chamber of adult mouse eyes, however, they grew, became erythropoietic, and were filled with lymphocytes. That the lymphoid cells are not intrinsic to the spleen, however, is suggested because spleen rudiments failed to become lymphoid when grafted into irradiated host animals. A relationship between spleen and thymus is suggested by the observation that in explants addition of splenic rudiments to thymus rudiments resulted in marked synergism. Interaction between spleen and thymus seemed to be specific inasmuch as synergism was not observed when spleen was replaced by lung, salivary gland, or limb mesenchyme. Morphogenetic interaction was seen also when spleen and thymus cells were mixed as suspensions and permitted to aggregate jointly.—NCI Monogr. No. 11: 23–33, 1963.

DURING THE course of experiments designed to elicit information on the morphogenetic processes involved in thymus differentiation (*1*, *2*), we were led to the hypothesis that the thymus may well represent the major primordium of the mammalian immunological system (*3*). The hypothesis at that time derived its support from a series of experimental observations of thymus differentiation in homologous tissue grafts (*3*), on a quantitative cellular characterization of mouse thymus lymphoid differentiation by use of a cell size analyzer (*4*), on a comparison of the timing of lymphoid differentiation during thymus development with lymphoid differentiation of other embryonic primordia (*5*), and on an analysis of the cellular origin of the thymus lymphoid cells (*2*).

The hypothesis had the merit of being readily subject to a series of experimental tests. It was predicted that embryonic thymectomy

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[3] With the technical assistance of Joseph Felsenstein, Morgan Fischer, Louis Kubai, and E. Mattie Morin.

should lead to a reduction of antibody-forming capacity. The beautiful experiments of Miller (*6, 7*), which demonstrate that neonatal thymectomy leads to reduction of the homograft response and to some degree also of antibody-forming ability in mice, are compelling evidence in this regard. The hypothesis further suggested that spleen differentiation would of necessity be a thymus-dependent process. The present paper demonstrates that spleen differentiation is not autonomous but depends on acquisition of cells of extrinsic origin. Another corollary of the hypothesis was that thymus and spleen should have the capability of influencing each other in their morphogenesis as studied *in vitro;* experiments reported in this paper demonstrate that spleen and thymus do interact in an apparently specific manner.

MATERIALS AND METHODS

Mouse embryos were obtained from crosses of BALB/c females with C3H males; embryonic age was determined by observation of vaginal plugs. (C × C3H)F_1 males were used as adult hosts in transplantation experiments.

In studies involving tissue interactions in thymus morphogenesis, thymus, lung mesenchyme, and limb mesenchyme were obtained from 12-day embryos, salivary gland mesenchyme and spleen from 13-day embryos. In all other studies, thymus and spleen were obtained from 13- or 14-day embryos.

Millipore filter assemblies, prepared by cementing 20 μ TH filter material to plexiglas mounts, were used for all cultures. To permit fusion of tissues prior to transfer, the top of the filter was used as a culture surface for 24 hours. Subsequent to fusion, tissues were temporarily removed, the filter assemblies inverted, and the tissues then placed in the well of the filter assembly. The methods have previously been described in detail (*2, 8*).

The tissue culture medium consisted of Eagle's basal medium supplemented with 1 percent chick-embryo extract (9-day), 10 percent horse serum, and 50 units per ml of penicillin and streptomycin. Cultures were incubated at 37.5° C in a water-saturated atmosphere of 5 percent CO_2 in air.

Dissociation of tissues to the cell level was achieved as follows: Tissues were cut into fragments not exceeding 0.3 mm in diameter, incubated at 37.5° C in calcium- and magnesium-free Tyrode's solution (CMF), then incubated for 15 minutes in a mixture of 1 part pancreatin 4× U.S.P. (Viokase) and 1 part crystalline trypsin prepared as a 1 percent solution in CMF, rinsed briefly in CMF, transferred to standard tissue culture medium, and mechanically dispersed in small tubes by controlled, vibrator-produced agitation (*9, 10*).

Transplantation of tissues to the anterior eye chamber was performed in standard fashion, sodium pentobarbital (Nembutal) serving as anesthesia. In experiments involving X irradiation, mice were irradiated on a rotating platform and given total body irradiation of 850 r, delivered as 140 kvp, 5 ma, 3 mm Cu X rays at a dose rate of 62.5 r per minute. Control animals were sham-irradiated.

Cultures were fixed in Zenker-acetic, transplants were fixed in Bouin's solution. Material was sectioned at 5 to 7 μ and stained with hematoxylin and eosin or with May Gruenwald-Giemsa. Cell suspensions prepared from living tissues were also examined in a quantitative manner, with a Coulter counter to obtain cell number and distribution of cell sizes; normal thymus (*4*) served as a standard for comparison.

RESULTS

Spleen Differentiation *In Vitro*

Spleen rudiments from 13- or 14-day embryos were isolated and cultured in standard filter-well cultures for varying periods. The method of filter-well culturing was chosen because of its demonstrated ability to support lymphoid differentiation of embryonic mouse thymus (*8*). Under these conditions the spleen rudiments failed to develop further, and after 1 week began to become necrotic in part. When spleen was isolated under conditions permitting spreading at an interface, fibroblastic-type outgrowth was observed, but characteristic lymphoid development did not occur. Preliminary observations of behavior of spleen explants taken from later-stage embryos and from mice of various ages suggest that morphogenesis is not much improved until at least 2 weeks of age, and that lymphoid differentiation of spleen cannot be achieved with embryonic or neonatal spleen with the methods employed (*11*).

The failure of embryonic spleen explants to become lymphoid *in vitro* might be interpreted either as a reflection of poor technique or, alternatively, as a reflection of the intrinsic inability of spleen at this stage to self-differentiate. To provide a more favorable environment for spleen differentiation, spleen rudiments from 13- or 14-day embryos were grafted to the anterior eye chamber of isologous adult male mice and grown for from 5 to 9 days. Such grafts grew well, and developed into large, red masses, which on histological examination proved to be composed of typical erythropoietic areas and central lymphoid regions (fig. 1). Lymphoid development was seen in all grafts, in contrast to the universal absence of lymphoid differentiation *in vitro*.

The results obtained in transplants suggested that either the host environment was able to support spleen lymphoid morphogenesis by providing a more favorable environment, or, on the other hand, that the observed lymphoid development was the result of host cells populating the graft. To distinguish between these alternatives, host animals were irradiated with 850 r total body irradiation 1 day prior to transfer of an embryonic spleen rudiment. Thymus rudiments were transplanted as control tissue; in general, each animal received a spleen rudiment in one eye, a thymus rudiment in the other. Grafts were recovered after 1 week and examined histologically. Spleen grafts had grown as well as grafts made into unirradiated animals, and showed normal erythropoietic development; on the other hand, they were devoid of lymphoid cells (fig. 2). Thymus grafts also had grown well and had developed into typical lymphoid organs. It was known from previous experiments that thymus lymphoid differentiation occurred in the absence of a host cellular contribution (*2*); the results obtained in the grafts to irradiated hosts support that conclusion and at the same time demonstrate that the irradiated host animals provided an environment with the capacity to support lymphoid differentiation. Thus the absence of spleen lymphoid differentiation in grafts to irradiated animals is most readily explained by the suggestion that the spleen grafts normally sequester cells of extrinsic origin.

Interaction of Spleen and Thymus *In Vitro*

In view of the postulated relation between spleen and thymus, and the demonstrated inability of obtaining spleen lymphoid differentiation *in vitro*, spleen from 13- or 14-day embryos was combined with thymus from 12-day embryos and allowed to develop for from 7 to 35 days. Spleen and thymus rudiments, grown separately, served as controls. As discussed spleen by itself failed to develop. Thymus by itself developed into a lymphoid organ, reaching its peak differentiation at about 12 to 14 days, followed by a gradual change to nonlymphoid cell types. Its overall growth was limited, the explant remained spherical, and the internal organization of the explant showed a fairly uniform distribution of the various cell types. Spleen-thymus combinations grew well, became considerably larger than thymus explants, became quite asymmetrical in appearance, and remained lymphoid for more than 5 weeks. The internal organization of the explant showed a subdivision of the lymphoid cells into pockets separated from each other by bands of connective tissue (fig. 3).

To test for the specificity of the effect exerted by spleen on the thymus explant, various other mesenchymes were used in combination with 12-day thymus rudiments. Salivary gland mesenchyme, lung mesenchyme, and limb mesenchyme, when combined with thymus, fully encapsulated the thymus rudiment but failed to exert any obvious effect on lymphoid morphogenesis.

To test for the origin of the lymphoid cells observed in spleen-thymus combinations, chick spleen was used in place of mouse spleen. When chick spleen (9-day embryo) was combined with mouse thymus (12-day embryo), the lymphoid elements which could be clearly classified appeared to be of mouse origin. It was not possible to distinguish and classify accurately the stromal elements appearing in these cultures.

The experiments involving spleen-thymus interaction suggest that the spleen exerts a distinct effect on thymic morphogenesis, an effect which is not duplicated by a variety of other mesenchymes tested. While a reciprocal effect of thymus on spleen is implied by the increased stromal proliferation observed in spleen-thymus cultures, critical tracing of the source of the stromal elements is still lacking.

To gain further information on spleen-thymus interactions, 14-day spleen rudiments and 14-day thymus rudiments were dissociated to the cell level and allowed to reaggregate as pure spleen, pure thymus, or spleen and thymus. When spleen cells reaggregate, they form characteristic reaggregates that fail to develop further. When thymus cells reaggregate, they form reaggregates which can, on further cultivation *in vitro* or in transplant, become typically lymphoid. When spleen cells and thymus cells are mixed and allowed to reaggregate jointly, they form heterotypic aggregates having a characteristic mottled appearance. On further cultivation they develop into complex organized structures, with lymphoid follicles enmeshed in a network of stromal elements. Mixed reaggregates thus exhibit to an even more pronounced degree the synergistic interaction observed when spleen and thymus rudiments are simply fused upon explantation.

DISCUSSION

It is important in these experiments to distinguish critically between established facts and postulated events. The study of spleen in explant, in transplants, and in transplants into irradiated animals establishes that the spleen fails to become lymphoid in the absence of critical cellular contribution of the host, but it does not establish the fact that it cannot do so under perhaps more favorable conditions; nor does it exclude the possibility that in due time it can become lymphoid *in situ*, *in vitro*, or in transplant, simply not yet having become sufficiently mature to do so. That host cells can migrate into an embryonic spleen, as suggested by our experiment, does not prove that this is the normal origin of spleen lymphoid cells. Finally, even if the normal origin of spleen lymphoid cells is indeed by immigration, it does not establish the fact that the cells in question originate in the thymus.

On the other hand, the hypothesis of thymic origin of splenic lymphoid cells is certainly attractive, for it readily explains the results obtained in experiments of isolated and grafted spleen, and in experiments involving spleen-thymus interactions *in vitro*. There is additional support for this hypothesis from the observations by Miller on neonatally thymectomized mice (*6*, *7*). In such animals, the spleen fails to develop further, and the histological appearance of a spleen several weeks after thymectomy is quite similar to the appearance or spleen seen in our cultures.

The specificity of spleen-thymus interaction can also be considered in this respect. One might well have predicted a generalized mesenchymal effect on the development of complex organization of a lymphoid system, but the specific spleen contribution to the system suggests a more meaningful relationship. Moreover, one must keep in mind that while spleen-thymus interaction *in vitro* has been demonstrated, spleen-thymus interaction of the kind shown in these experiments clearly cannot occur *in situ*, inasmuch as there is no physical proximity between these organs during development.

In view of the considerable information available on reaggregation patterns in mixed suspensions (*9*, *12*), the appearance of reaggregated spleen-thymus mixtures deserves comment. The reaggregates do not sort out in the manner normally expected of two different tissue types, but rather organize as an integrated lymphoid organ. Again, the results of our reaggregation experiments are suggestive of a meaningful relationship between spleen and thymus. If the spleen normally sequesters cells and permits their reorganization within its confines, then the results obtained in the present experiments may be interpreted directly, but such interpretation is, for the moment, premature.

It has been postulated that the thymus and spleen are the collaborators yielding a mature antibody-forming system (*3*, *7*). That thymus-spleen interaction can occur *in vitro* and result in complex lymphoid differentiation argues for the suggestion that spleen-thymus cultures should be capable of producing antibodies in tissue culture [*cf.* (*3*)]. Experiments designed to explore this suggestion are in progress.

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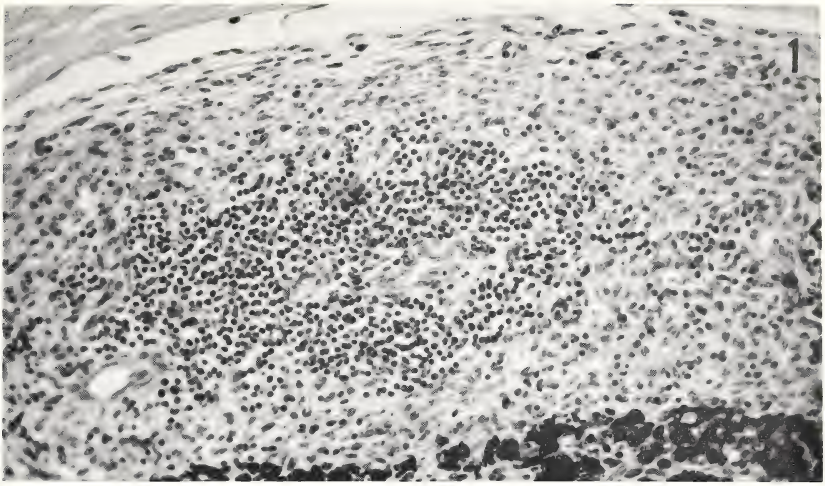
PLATE

PLATE 2

FIGURE 1.—Implant of 14-day embryonic spleen rudiment grown for 7 days in anterior eye chamber of a normal adult mouse. *Note* deeply staining small lymphocytes in *center* of graft.

FIGURE 2.—Similar implant grown for 7 days in anterior eye chamber of a mouse previously irradiated with 850 r. *Compare* with figure 1. *Note* absence of lymphoid cells in graft.

FIGURE 3.—Culture of combined splenic and thymic rudiments after 9 days *in vitro.* Lymphoid follicles are seen enmeshed in splenic reticular material.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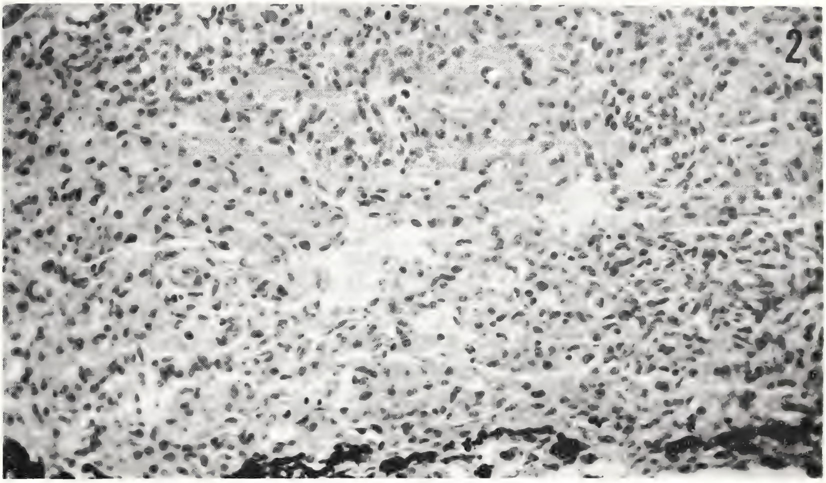
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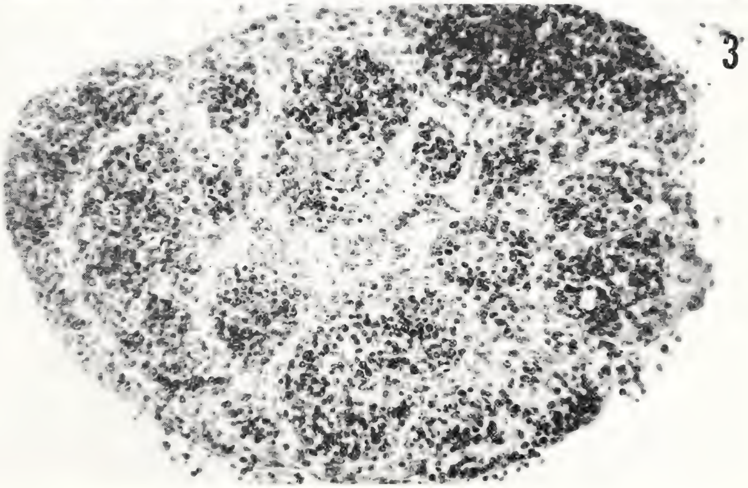
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DISCUSSION

JAMES D. EBERT, Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland

Interest in the embryonic development of the cells responsible for immune mechanisms, sparked by the discovery of actively acquired tolerance and maintained in large part by studies of the graft-versus-host reaction, has been for nearly a decade a prime target of research. Yet despite the intensity with which studies have been conducted, our knowledge of the embryonic origins of these cells has remained limited.

Limited, too, is our knowledge of the role of that enigmatic structure, the thymus. As summarized effectively by Martinez, Kersey, Papermaster, and Good (Proc. Soc. Exper. Biol. & Med. 109: 193–196, 1962), the thymus is rich in lymphocytes, yet is not truly effective in the production of antibodies; it is maximal in size at an early age when immunological capacity is minimal. Also, they point out, there is a high incidence of thymoma in patients with agammaglobulinemia.

Viewed against this background, the observations reported so effectively by Auerbach, as well as those of Martinez *et al.* and Miller, mark the beginning of what may prove a major stride in this field. In view of the outstanding possibilities of the work described, therefore, it seems appropriate that the following questions be addressed to the speaker:

1) Martinez *et al.* demonstrated, in agreement with Miller, that in certain strain combinations of mice thymectomy of the host resulted in prolonged survival of homografts. However, in other strain combinations, thymectomy has no significant effect. Moreover, early thymectomy served to depress immunological capacity sufficiently to overcome a weak histocompatibility difference, but not a strong difference. Do you believe, therefore, that the evidence warrants a generalization as strong as yours appeared to be concerning the effects of thymectomy?
2) Your description of the experiments in which spleen was grafted into the anterior chamber of the eye raises two questions: First, in the homologous combinations, a host-versus-graft reaction was described. Did you also observe a graft-versus-host reaction? And, second, in the isologous combinations, how can you rule out the possibility of a humoral factor in supporting splenic differentiation in contrast to your preferred hypothesis of "seeding" by migrating host cells? The irradiation experiments do not resolve the question. Have you planned experiments with labeled grafts or hosts, *e.g.*, the use of chromosome markers or isotopes?
3) In how many, and which, tissues have you sought lymphocytes before they are observed in the thymus? I am struck by the fact that in the chick embryo, though lymphocytes appear in the spleen relatively late in development (as in the mouse), thus conforming to the idea advanced here of a thymus-spleen interaction, there are substantial numbers of lymphocytes in other organs much earlier.

Comments by Dr. Auerbach in Response to Questions of Dr. Ebert

1) The differences observed between various strains of mice, as well as the less than absolute effects of thymectomy in Miller's experiments, could well be explained as the result of performing the operation at too late a point in development. You may recall our initial suggestion was that *embryonic* thymectomy should lead to loss of immunological competence. Different strains of mice vary in the degree of maturity at time of parturition. Recent experiments of ours involving transplantation of spleen at various ages of development suggest that spleen lymphoid autonomy in $(C \times C3H)F_1$ mice begins before birth.

2) While we have not described homologous transplants in the present paper, such experiments have indeed been performed. In these a homograft reaction was observed as judged by the localization of lymphoid elements within the grafted rudiment. Experiments designed to determine the extent of graft-versus-host response as distinguished from host-versus-graft response are now being analyzed.

We cannot unequivocally rule out a humoral factor in the transplant experiments; your point is well taken. However, the combined information from spleen grafts, synergistic interaction *in vitro*, and the thymus lymphoid differentiation in irradiated animals does not argue for a dominant role of a humoral factor. Furthermore, when lymphoid cells first appear in transplanted spleen rudiments they are all of the mature type; early stages of lymphoid differentiation are not seen.

3) Small numbers of lymphoid cells may well be present in a number of embryonic organs such as the lung and the liver. However, the thymus appears to be the only *major* lymphoid organ in mouse embryos.

REAGGREGATION AND ORGANOTYPIC REDEVELOPMENT OF DISSOCIATED EMBRYONIC CHICK LUNG CELLS IN SHORT-TERM CULTURE[1, 2]

JOHN W. GROVER,[3] Boston Lying-In Hospital, Boston, Massachusetts

SUMMARY

A technique for the enzymatic dissociation of embryonic chick lung is described, with subsequent reproducible reaggregation and culture of the viable dissociated cells. The effectiveness of dissociation and reaggregation diminishes with increasing embryonic age. An optimum age for reconstructive histogenesis is observed at 11 and 12 days of incubation; prior to this, though aggregation is complete, histogenesis is not totally representative of the original tissue. Older tissues show a diminishing capacity for both reaggregation and histogenesis. At 12 days of embryonic age, epithelial and mesenchymal cells of the lung may be dissociated separately; when reaggregated in varying proportions, histogenesis is dependent on a proper balance between the two cell types and is relatively independent of time relationships in culture. These phenomena are discussed in relation to the fundamental biologic principles of intercellular adhesion, cell motility, selective segregation and adhesion, and structure formation. To invoke inductive processes, organizers, field effects, or the actions of diffusible substances is considered less useful in an understanding of these experimental observations than the concept of self-organization.—NCI Monogr. No. 11: 35–50, 1963.

A DECADE AGO, Moscona reported the development of techniques for the dissociation of embryonic organs and the reaggregation of the cells in tissue culture (*1*). However, only in the last several years have reproducible methods become available for the systematic study of cellular interrelationships and behavior in short-term reaggregation cultures. One such method (*2*) has been used to evaluate the histogenetic capabilities of dissociated chick-embryo lung cells over a broad spectrum of ages, and to examine the mesenchymal-epithelial relationships of reaggregating cells at the embryonic age that was found to be optimum for histotypical reorganization. These and other studies of the behavior of embryonic chick lung cells during dissociation and reaggregation have been reported previously (*3*, *4*).

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[1] Presented at The 13th Annual Meeting of the Tissue Culture Association, Washington, D.C., May 29–31, 1962.

[2] This study was made under a postdoctoral grant CF-10,012 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] The author wishes to express his gratitude to Dr. Honor B. Fell, F.R.S., Director of The Strangeways Research Laboratory, Cambridge, England, for her inspiration and assistance and for the facilities of the laboratory where this work was performed.

MATERIALS AND METHODS

Dissociation of tissues.—Embryonic lung tissue of the desired age is obtained aseptically, washed in calcium- and magnesium-free Tyrode's solution, and incubated 30 minutes in trypsin solution (Difco 1:250 trypsin, 0.5% in calcium- and magnesium-free Tyrode's solution) on a slowly turning roller-tube apparatus at 38° C. Partial mechanical separation is obtained by aspiration with a Pasteur pipette, after the addition of horse serum, to protect the cells from mechanical damage. The suspension is washed by centrifugation at 150 × *g*, resuspended in collagenase (Worthington Laboratories, Freehold, N.J.; 0.05% collagenase dissolved in a solution containing NaCl, 8.00 g/liter, KCl, 0.40 g/liter, and glucose, 1.00 g/liter), and incubated for 15 minutes. Horse serum is again added, and final dissociation carried out by aspiration. Undissociated particles, if present, are removed by slow centrifugation. The suspended cells are washed by centrifugation at 150 × *g*, and resuspended in 50 volumes of fluid culture medium to give an approximate cell count of 10,000 cells per mm^3. The viability of the cells is estimated by a check of eosin uptake (*5*).

Reaggregation and culture.—The fluid culture medium used is the exudate obtained from an embryo extract-plasma clot which is broken up and centrifuged through glass beads to remove the fibrin. The suspended cells are cultured as individual drops on the inner surface of an inverted petri dish cover, which is immediately turned upright. Hanging drops are thus produced, within which the cells settle and aggregate on the meniscus rather than adhere to the glass surface. Multiple preparations are placed in a suitable humidified chamber and gassed with 95 percent oxygen and 5 percent carbon dioxide and incubated at 38° C for 24 to 48 hours.

Preparation of epithelium and mesenchyme.—After tryptic digestion of 11- or 12-day lung, dissociated mesenchymal cells are separated from intact epithelial tubules by slow centrifugation. The two preparations are washed and incubated separately with collagenase, as outlined previously, and resuspended in fluid culture medium. Pure suspensions are cultured directly, while mixed suspensions are prepared by a combination of proportionate amounts of the two types before culture.

Histology.—The reaggregated specimens and suitable control tissues of normal embryonic chick lung are fixed in 25 percent acetic alcohol, sectioned, and stained with Delafield's hematoxylin and chromotrope stains. Each experimental result was reproduced at least 3 times.

RESULTS

Normal Embryonic Lung

The normal embryonic lung development progresses in an organized fashion after the appearance of lung buds from the primordial trachea at about 4 days of incubation. At 7 days (fig. 1), the tiny lungs consist of secondary epithelial subdivisions of the major bronchi embedded in a dense, avascular mesenchyme. The tertiary subdivisions, or parabronchi, are well established by 11 to 12 days (fig. 2) and are surrounded by a prominent basement membrane. They are supported by a much looser mesenchyme, and are arranged in lobules with the developing blood vascular system. After this time, the complexity of the lung rapidly increases, and by 18 days (fig. 3) the lung is a highly developed lobular system based on the parabronchus, its air capillary outgrowths, and their intimate interdigitations with the greatly proliferated vascular network. Smooth muscle structures have differentiated near the lumen of the unit, and the loose mesenchyme characteristic of development at 11 and 12 days is much diminished.

Dissociation

Dissociation of these tissues is a function of their embryonic age. The younger lung, age 7 through 14 days, dissociates completely while older tissue is progressively more difficult to break down into isolated cells. At 11 and 12 days of age, trypsin dissociates only the mesenchyme (fig. 4) and leaves relatively intact parabronchi, which subsequently dissociate completely in collagenase. The final suspension of every preparation is free of undissociated structures and consists largely of isolated single cells (fig. 5). Fewer than 5 percent of cells in all preparations were permeable to eosin, consistent with a high degree of viability.

Aggregation

The effectiveness with which dissociated lung cells aggregate is also related to their embryonic age. From 7 through 12 days of development, reaggregation is complete, and all the cells in each drop form a single aggregate. The older the cells, the less well they aggregate. At 18 days, approximately 50 percent of the cells remain in free suspension.

Histogenesis

After 7 hours' culture, 11-day-old dissociated cells form a loose aggregate with no reorganization of cells with respect to type (fig. 6). Eighteen hours' culture allows the gross sorting of cells into epithelial and mesenchymal groups within the aggregate (fig. 7), without typical architectural reconstruction. Tubules begin to re-form after 24 hours (fig. 8), and both tubules and mesenchyme are typical of the parent tissue at the end of the 48-hour culture period (fig. 9). There is no mitotic activity during the first 24 hours, but it resumes in both epithelium and stroma during the second 24 hours.

A comparative study of the histogenetic capabilities of dissociated cells of different ages under these conditions shows that 7-day-old cells aggregate and organize readily into mesenchyme covered with epithelium, but there is scant tubular or vascular re-formation (fig. 10). Twelve-day cells show well-formed branching and interconnecting epithelial tubules as well as vascular spaces in a typical spatial relationship (fig. 11). Fourteen-day-old tissues are only slightly more developed than at 12 days and are easily and completely dissociated. However, 14-day cells do not aggregate as effectively as 12-day cells, and they demonstrate a diminished capacity for histotypical reconstruction (fig. 12). The re-formed tubules and other structures, though similar to the parent tissue, are not as perfect as those formed by 12-day-old cells. After 17 days of age, the dissociated lung cells that reaggregate do not form structures well (fig. 13). Poorly defined tubules and atypical epithelial structures, with little mesenchymal or vascular reorganization, are characteristic findings when dissociated cells of this embryonic age are reaggregated and cultured.

Twelve-day-old epithelial cells cultured alone for 48 hours demonstrate no histogenetic activity. The cells remain loosely grouped in clumps and rosettes without supracellular organization (fig. 14). The addition of a small amount of mesenchyme permits the formation of many tiny mesenchymal aggregates, each surrounded by a single layer of epithelium (fig. 15). No tubules form, and epithelium not in contact with mesenchyme remains unaltered. As the proportion of mesenchyme is increased, the aggregate size increases, until an optimum proportion is reached, at which time the expected reaggregation and reorganization occurs (fig. 16). When mesenchyme far exceeds epithelium, small epithelial clumps form, buried within reaggregated mesenchyme after 24 hours' culture. These clumps are apparently unable to establish contact with one another and, rather than tubules, cystic structures are formed after 48 hours (fig. 17). No epithelial cells appear on the surface of such aggregates.

Twelve-day epithelial cells aggregated and grown in pure culture for as long as 19 hours retain their capacity for interaction with freshly dissociated mesenchymal cells (fig. 18). However, the discrete aggregates of 12-day epithelium and mesenchyme that have formed after separate culture for 24 hours do not interact and remain unchanged after 24 hours' combined mesenchymal-epithelial culture in the same drop. In the latter condition, no close physical contact between the two aggregates is established.

DISCUSSION

Both trypsin and collagenase are required for the complete dissociation of embryonic chick lung at all ages, as the basement membrane of the tubules is resistant to trypsin. The increasing resistance of older tissues to digestion is probably related to the stabilization of collagenous components of the basement membrane and to the formation of stronger intercellular attachments as full differentiation is approached.

Older cells may have an intrinsic disability for reaggregation and histogenesis as a consequence of aging. However, it is also likely that the older cells have been subtly damaged as they dissociate, without loss of viability. Moscona (*6*) has recently confirmed observations relating a diminishing effectiveness of aggregation with increasing embryonic age, using chick embryo neural-retina cells. Histogenesis by the older cells may also be affected by chance factors, since the number of different cell types within the aggregates are increased during continued differentiation. The statistical likelihood that similar cell types will encounter each other in sufficient number to form typical structures is thus reduced. Moscona (*7*) described a similar situation when he observed that in reaggregations of mixed cell lines, typical structures were not formed when the proportion of a given cell type was lowered below a critical level, which indicated that the cells were not present in sufficient number to selectively reorganize. The relatively simple histogenesis that occurs in the cultures of 7-day-old cells is believed to reflect the underlying simplicity of this tissue, which consists predominantly of epithelium and mesenchyme. However, the failure of the epithelial cells to re-form tubules resembling secondary bronchi in these aggregates remains unexplained.

The progressive changes in structure brought about by alteration of the proportion of mesenchyme to epithelium at 12 days demonstrate their obligatory interdependence during histogenesis. These observations recall the work of Rudnick (*8*) and Grobstein (*9*) which demonstrated mesenchymal-epithelial interdependence during differentiation and growth of lung and salivary gland under experimental conditions. It is likely that the presence of a suitable number of mesenchymal cells provides a milieu in which epithelial cells can express their innate histogenetic capacity. The mesenchyme may provide a substrate for cell movement and, in intimate association with the epithelium, may resynthesize specific intercellular substances required for histogenesis. Herrmann (*10*) has demonstrated an obligatory interdependence related to the enzyme content of corneal epithelium and stroma. A metabolic interaction between the cell types during epithelial regeneration was observed, and similar mechanisms might be operative in chick-embryo lung.

Curtis has proposed that the duration of culture is important in the reorganization of dissociated amphibian cells (*11*). It was believed that the differential stabilization of cell types during the first few hours of culture determined their localization in the aggregate. In contrast, the present work indicates that 12-day embryonic chick-lung epithelial cells retain their capacity for interaction with mesenchymal cells in a typical way for as long as 19 hours. This is longer than the critical time observed by Curtis, and more than the time required for gross epithelial segregation in hanging-drop culture. The failure of mesenchymal and epithelial cells reaggregated separately for 24 hours to interact in combined culture is probably related to the lack of close physical contact between the aggregates.

The processes of reaggregation and reconstructive histogenesis conform to several simple fundamental principles. The first and primary prerequisite is for the mutual adherence of all cells. In these preparations, adhesion is temporarily aided by the passive contacts among the cells as they settle on the meniscus of the drop. More selective and more permanent cell-to-cell bonds are subsequently formed as the tissue reorganizes.

A second prerequisite is cell motility. The cells are in vigorous and constant movement during the first 24 hours when segregation occurs according to cell type. Cell movements, either singly or in groups, continue during the second 24 hours when histotypical structures are reorganized from the amorphous masses of segregated cells. It is not known whether these cell movements are random or are directed, but it is likely that both random and directed cell movements occur at different phases of reconstruction.

The third requirement is a capacity for selective or specific segregation and adhesion of cells within the aggregate. It is likely that segregation of cells by type depends on their surface characteristics, probably in relation to extracellular materials.

The fourth, and perhaps least understood requirement, is the ability of cells to reorganize into histotypical structures once they have grouped themselves by the processes of adhesion, movement, and segregation. This ability is obviously closely related to the interactions between mesenchymal and epithelial cells within the tissue.

Field effects, related to oxygen or metabolite diffusion gradients do not seem to be implicated in these processes, since all cell types, with the possible exception of vascular endothelial cells, may appear at any location within or without the aggregate, depending on the mesenchymal-epithelial cell balance. Inductive processes, in the embryonic sense, are apparently not applicable. By reconstitution of histologic structures, the cells are reproducing structural forms that existed prior to the dissociation of the tissue. They have therefore already expressed some part of their previously induced developmental capacity, and to repeat these forms no further inductions need be invoked. The rapidity with which histologic reconstruction takes place, its independence of cell multiplication, and its variable form, as influenced by the balance between epithelial and mesenchymal cells all provide additional evidence that embryonic induction in the usual sense is not involved. These facts strengthen the assumption that dissociation of tissue does not reverse the induced capabilities of the cells. The observation that intimate intercellular contacts are required indicates that diffusible substances are not operative, either in an inductive or a general sense.

My observations describing cellular behavior in culture are consistent with the concept of self-organization, proposed by Weiss and Taylor (*13*). These processes are independent of external influences, such as inducers or organizers, and are dependent only on individual cell characteristics and intercellular relationships. The concept of self-organization, though incomplete, provides the best current theoretical basis for understanding the behavior of embryonic cells in these experimental conditions and, by extension, may lead to more knowledge of normal growth and differentiation in the intact embryo and of abnormal cellular behavior in neoplasia.

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PLATE 3

Histologic sections and smears in the plates were stained with Delafield's hematoxylin and chromotrope stains.

FIGURE 1.—Seven-day embryonic chick lung. Secondary bronchi are surrounded by relatively dense and poorly vascularized mesenchyme. × 270

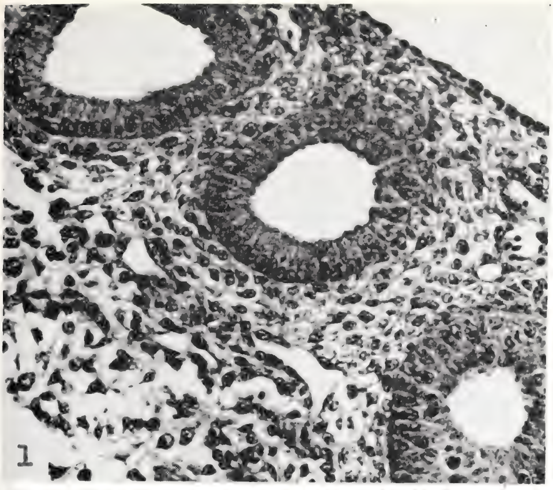
FIGURE 2.—Eleven-day embryonic chick lung. Tertiary or parabronchi are surrounded by loose mesenchyme, and are equidistant from the developing blood vessels. × 270

FIGURE 3.—Eighteen-day embryonic chick lung. Epithelial outgrowths of the parabronchus form well-defined air capillaries. Loose mesenchyme is replaced by blood vessels, and smooth muscle appears near the central lumen. × 270

FIGURE 4.—Eleven-day embryonic lung digested with trypsin for 30 minutes. Dried smear. Mesenchyme has been dissociated, leaving parabronchial epithelium intact inside basement membrane. × 105

FIGURE 5.—Eleven-day embryonic lung digested with trypsin followed by collagenase. Dried smear. Tissue has been completely dissociated. Loose clumping of cells occurs as smear dries. × 270

FIGURE 6.—Portion of aggregate formed by dissociated 11-day lung cells after 7 hours' culture. Cells are loosely associated, and there is little segregation by type. No mitotic figures. × 270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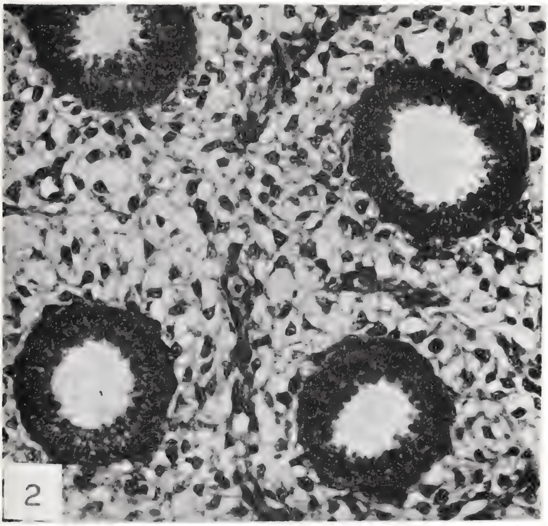
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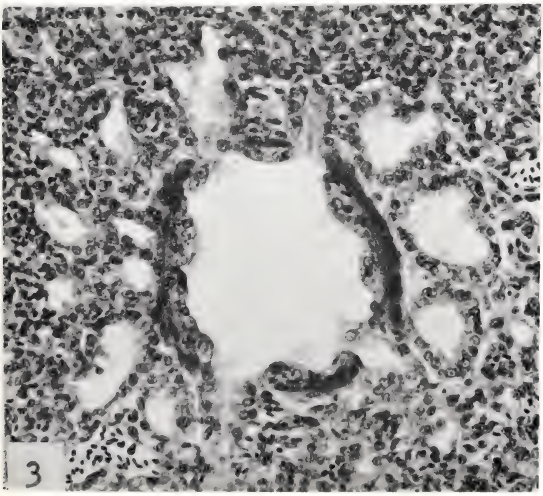
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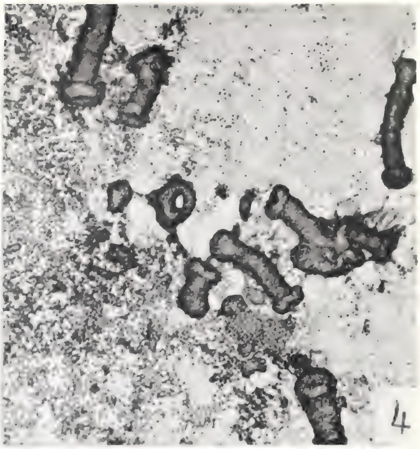
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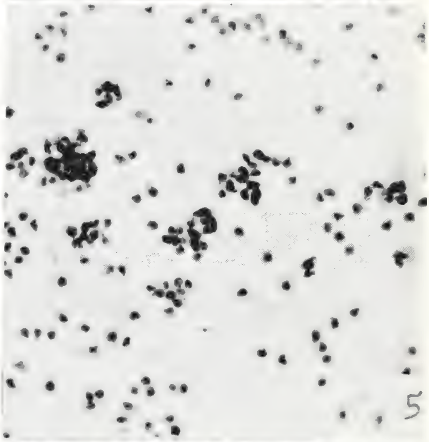
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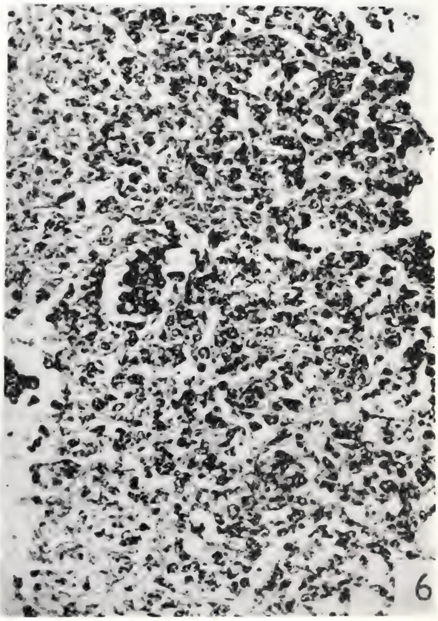
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Plate 4

Figure 7.—Portion of aggregate formed by dissociated 11-day lung cells after 18 hours' culture. Definite segregation by type has occurred, the cluster of epithelial cells stains densely, and mesenchyme is compact. Mitoses are rarely seen. × 270

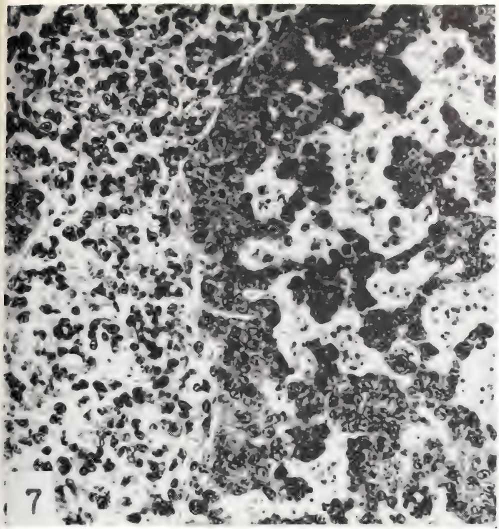
Figure 8.—Portion of similar aggregate after 24 hours' culture. Epithelial tubes resembling those in intact 11-day lung have formed, but the mesenchyme remains compact. Mitotic activity is increasing. × 270

Figure 9.—Portion of similar aggregate after 48 hours' culture. Well-formed epithelial tubes are present, and mesenchyme is more typical of 11-day lung. × 270

Figure 10.—Portion of 48-hour aggregate of dissociated 7-day embryonic lung cells. Aggregate is coated with a tall columnar epithelium, but no tubes have formed within the mesenchyme. × 270

Figure 11.—Portion of 48-hour aggregate of dissociated 12-day embryonic lung cells. Branching epithelial tubes surrounded by characteristic mesenchyme are present, and vascular structures in their proper relation to epithelium and mesenchyme appear in *center* of figure. × 270

Figure 12.—Portion of 48-hour aggregate of dissociated 14-day embryonic lung cells. Epithelial tubes, vascular structures, and mesenchyme are present, but are not as well formed as at 11 or 12 days. × 270



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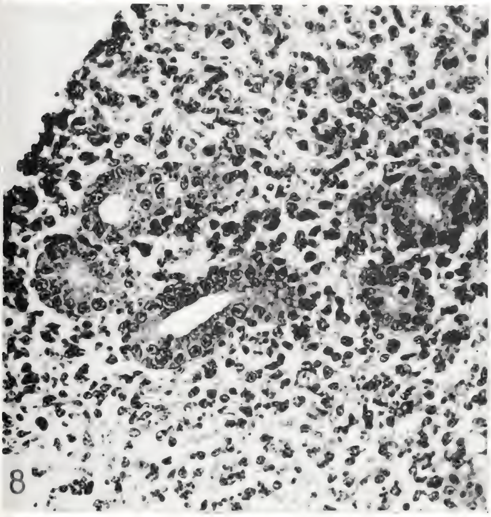

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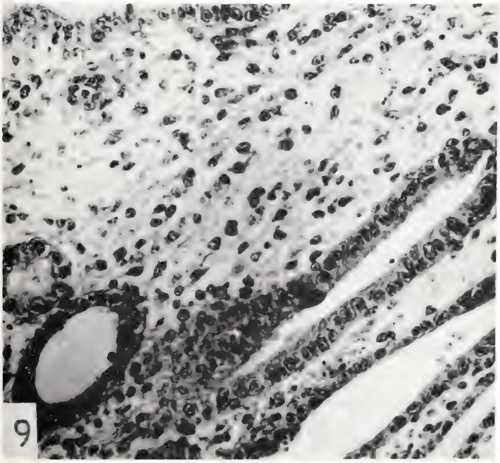

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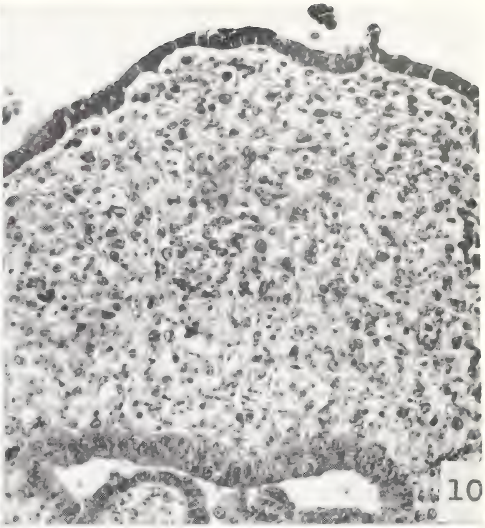

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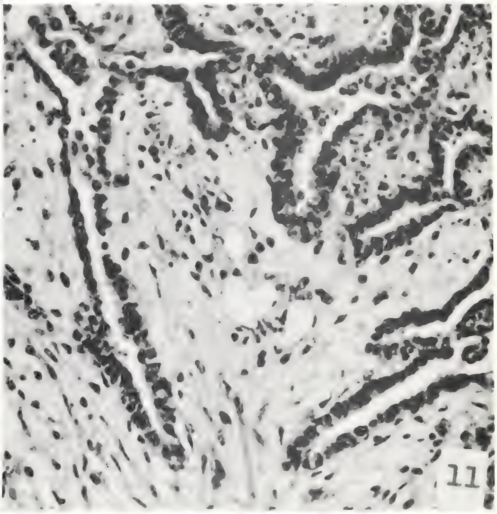

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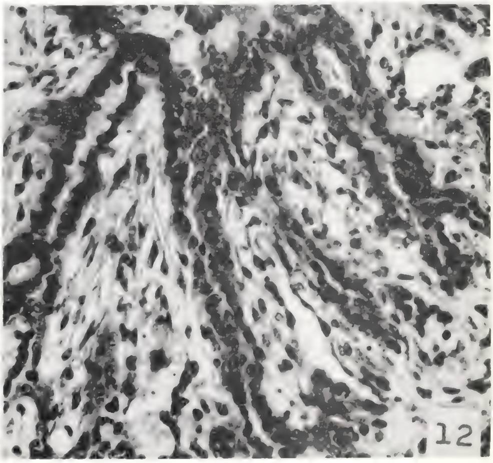

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Plate 5

Figure 13.—Portion of 48-hour aggregate of dissociated 17-day embryonic lung cells. The attempted histogenesis is abortive, and no typical structures are present. × 270

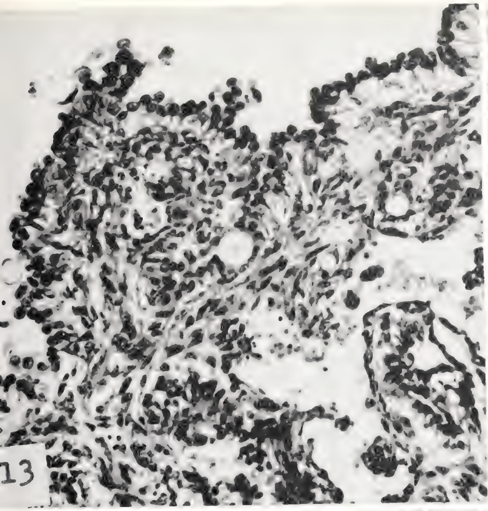
Figure 14.—Portion of 48-hour aggregate of dissociated epithelial cells from 12-day embryonic lung. Cells are loosely associated in clumps and rosettes without typical histogenetic activity. × 270

Figure 15.—Portion of 48-hour aggregate of dissociated epithelial cells from 12-day embryonic lung, with a small amount of dissociated mesenchymal cells added at time of culture. Small clumps of mesenchyme coated with epithelium have formed. Epithelial cells not in close contact with mesenchyme are inactive. × 270

Figure 16.—Portion of 48-hour aggregate of dissociated epithelial cells from 12-day embryonic lung, with enough added mesenchymal cells to allow for typical reconstructive histogenesis. Tubes, mesenchyme, and vascular structures are present and are typical of the parent tissue. × 270

Figure 17.—Portion of 48-hour aggregate of dissociated cells from 12-day embryonic lung in which an overabundance of mesenchyme is present. Cystic epithelial structures have formed—a gross distortion of the usual tubular histogenetic pattern. × 270

Figure 18.—Portion of 48-hour aggregate of dissociated cells from 12-day embryonic lung, to which freshly dissociated mesenchymal cells were added after 19 hours' culture. Epithelium has interacted in a typical manner with mesenchyme. Mesenchymal clumps are covered with epithelium, and several tubules can be seen. × 270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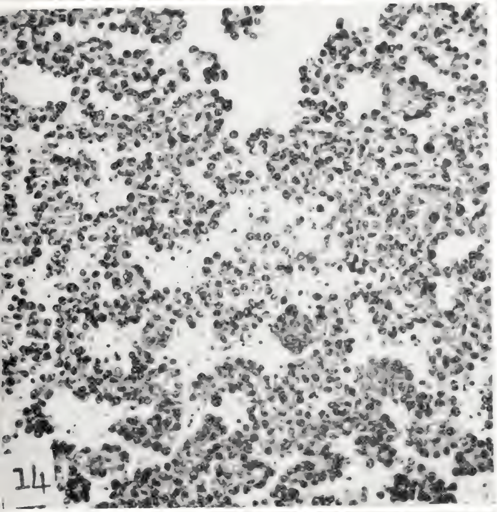
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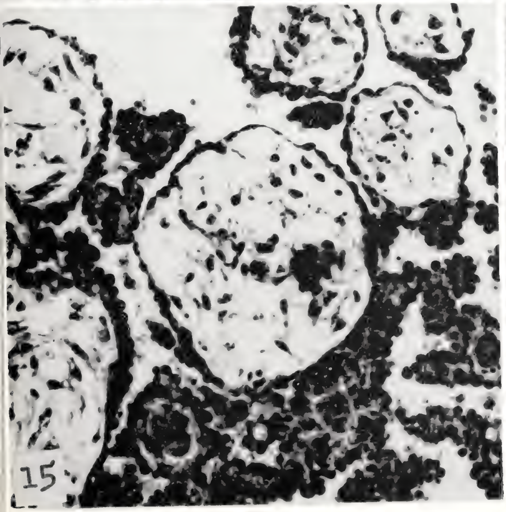
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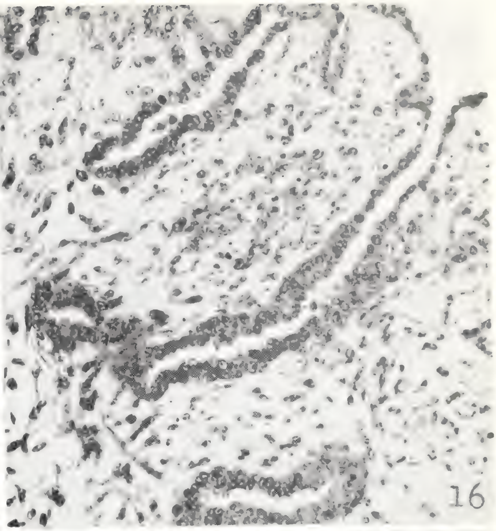
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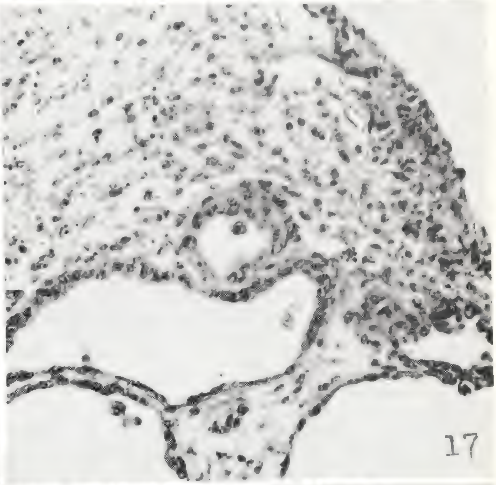
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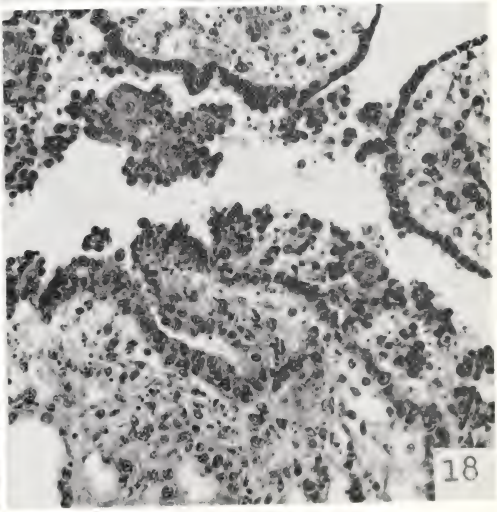
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DISCUSSION

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The experiments of Wilson (*1, 2*), Galtsoff (*3*), Roudabush (*4*), and Spiegel (*5*) with dissociated sponge and hydroid cells, of Holtfreter (*6, 7*), Townes and Holtfreter (*8*), and Steinberg (*9*) with dissociated salamander cells, and of Moscona (*10–12*), Weiss and Taylor (*13*), and Trinkaus (*14, 15*) with dissociated chick and mouse cells provide evidence for the following generalizations: 1) Dissociated cells exhibit differential affinities for cells and substrates; 2) under certain conditions mixed populations of dissociated cells may segregate and form organized systems of some complexity; 3) presumably the immediate locus of this behavior resides in the properties of the membranes of the interacting cells. However, what is still unclear to me is how much of the reaggregated cellular architecture is due exclusively to the reassembly of unchanged and essentially static cells, and how much requires inductive, regulative, or cell-to-cell interactions. In this critique, induction, regulation, and cell-to-cell interaction will be used interchangeably. These terms simply refer to those events that alter the differentiated state of the responding cells, events of which we as embryologists are totally ignorant [Holtzer (*16, 17*)].

Dr. Grover has shown how cells of the developing lung of different ages and degrees of complexity reaggregate under various conditions. The elegance of Dr. Grover's experiments stems from the fact that they are short-term ones and hence probably exclude the complications of *regeneration and regulation* that would be superimposed if the cells were cultured for a long period, say on the chorio-allantoic membrane [*see* (*13*)].

A dissociated 7-day lung reaggregated in a hanging drop forms a mesenchymal mass *covered* by epithelium. Dissociated 11- and 12-day lungs evidence tubules surrounded by mesenchyme. Older lungs, which prior to dissociation consisted of lobular systems, air capillary outgrowths, smooth muscle, and mucus-secreting cells, reorganize very poorly. Epithelial cells cultured alone do not form tubules; tubule formation requires an optimal proportion of epithelial to mesenchyme cells.

From these observations it is concluded that 1) segregation occurs according to cell types, and 2) histotypical structures appear in the reconstituted tissue by virtue of "self-organization." This self-organization is independent of external influences and is dependent only upon individual cell characteristics and intracellular properties. Of these two conclusions I believe that the experimental evidence warrants qualified acceptance of the first, but the evidence for the second is much less convincing and invokes concepts I do not understand.

I think Dr. Grover would agree when I suggest that the most compelling demonstration of segregation according to cell type would be with older material. If smooth muscle aggregated with smooth muscle, mucus secreting with mucus, ciliated epithelium with ciliated epithelium, etc., then one could say homologous cells adhered to one another. But, as stressed in Dr. Grover's report, this did not happen. The closest the experimental evidence came to a demonstration of adherence of like cells was with 7- and 11-day lung which really involves only two kinds of cells—epithelium and mesenchymal. With this material, one judges from the photographs, only a modest degree of histotypical reorganization occurs and one hardly unique to the lung. Along these lines, if graded numbers of intestinal or kidney epithelial cells were substituted for lung epithelial cells, would the resulting aggregate be more or less lunglike?

In any event, if the dictum of like cells adhering is accepted then *histotypical reorganization must be excluded.* For the lung is not an aggregate of clusters of like cells. The lung is a 3-dimensional fabric where different kinds of epithelial cells form a sheet which in turn establishes intimate spatial relationships with endothelial, cartilage, and mesenchymal cells. For histotypical reorganization to occur it would be necessary to postulate selective adhesions between epithelial and mesenchymal cells as well as between epithelial cells.

If it is assumed that only those cells that were epithelial cells prior to the dissociation are the epithelial cells appearing after reaggregation, then we are faced with some interesting questions. An epithelial cell before dissociation confronts three microenvironments: Part of its cell surface is adapted to cohere with adjacent epithelial cells, part borders the lumen of the tubule, while its antipodal surface is associated with a basement membrane. The questions are: After isolation and during the compacting period, when the cell is presumed to be amoeboid, do the cells retain their tripartite differentiated cell membranes? Do the motile cells retain their polarity? If so, will the lumen surface adhere or repulse the basement membrane of another epithelial cell? Do mucus-secreting cells adhere to squamal epithelial cells? Would lung epithelial cells adhere to kidney epithelial cells? Would such polarity survive cell division? Equally fascinating would be knowledge of how the intracellular synthetic pathways know how and where to deliver their unique products so as to maintain this differentiated surface while the cell migrates.

Alternatively, one may assume that the polarity of the epithelial cell is not retained during the sorting-out period. Then an equal number of interesting questions arise. I would like to mention only one in some detail. If the polarity is lost, then it must be reacquired and my guess would be that it is reacquired *after interacting with other epithelial and mesenchymal cells.* If the lumen of the tubule forms secondarily from a solid mass of epithelial cells, such axial differentiation of a single cell must be due to inductive activities. Lumen formation may involve secretory activity at one pole and basement-membrane formation at the other pole of the cell. Weiss who has theorized so persuasively for "self-organization" has shown that basement-membrane formation of the skin of the tadpole involves an inductive interaction between epithelium and mesenchyme [Weiss and Ferris (*18*)]. This reacquisition of polarity would require intracellular reorganizations. Now, while nothing is known biochemically of how the chorda induces the nervous system or of how the spinal cord or notochord induces vertebral cartilage, one thing is certain—induction is eventually expressed by intracellular reorganizations of the synthetic pathways of the responding cells.

Until more is known of the large numbers of sequential inductive events operating to produce a lung, I think it premature to state that the reconstituted tissue is not in any way dependent upon cell induction. Likewise, until more is known induction cannot be ruled out on the basis of time. Some inductive acts may take minutes, others, days before their cytological consequences are expressed and detected under the microscope.

In conclusion, my hesitation in accepting the term "self-organization" is derived from the fact that this term is used as an explanation, whereas it is but a biased restatement of the experimental data. As a concept, it is a lineal descendent of the "field" concept in embryology. And just as the field concept obstructs experimentation in embryology so, I suspect, will the concept of self-organization. If we are to understand the behavior of cells interacting with their own kind, or interacting with heterologous cell types, we must observe the cells in action and not be lulled by popular slogans.

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STUDIES ON MOUSE CHIMERAS DEVELOPED FROM EGGS FUSED *IN VITRO*[1, 2]

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SUMMARY

Eight-cell mouse ova are suitable for experimental production of chimeric animals. After removal of the zona pellucida, 2 naked eggs were squeezed together in a small drop of medium suspended in liquid paraffin and subsequently cultured *in vitro* in Krebs-Ringer bicarbonate, plus bovine plasma albumin and glucose, for 24 or 36 to 40 hours. This method ensured a complete union of 2 eggs and formation of a double, giant, but completely normally built blastocyst. After transplantation of double blastocysts to pseudopregnant mice, normally built embryos developed in a high proportion of cases. Regulation of size takes place very early, since from the 6th day onward, the embryos fall within the range of normal variation in size. Of 16 young littered naturally or delivered by cesarian section, only 2 survived. Both were fertile males. One series of experiments involved fusion of eggs of dark and light genotype. Histological examination of the retina revealed a mosaic pattern in 7 of 9 newborn animals. The sex ratio among 16 born animals was: 2 females, 3 intersexes, and 11 males. The low incidence of intersexes and the preponderance of males suggest that the male component in some of the sex chimeras exerted a dominant effect on sexual differentiation.—NCI Monogr. No. 11: 51–71, 1963.

DEVELOPMENTAL BIOLOGY now has at its disposal several techniques by which chimeric tissues and organs can be obtained. These techniques are based on reaggregation of dissociated cells and on grafting experiments during embryogenesis. Experimental parabiosis of avian embryos or transplantation of hematopoietic tissue in mammals gives rise to blood chimeras. These three ways of experimentation enable the chimeric state to be achieved at three successive levels, namely, at the tissue level, at the organ (or part of the body) level, and at the systemic level. To obtain a higher degree of chimerism, which would involve all or nearly all systems of an individual, it is necessary to unite the genetically

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[2] I am grateful to the National Cancer Institute, whose financial support to the Tissue Culture Association made it possible for me to participate in this symposium.

[3] My sincere thanks to Professor F. W. R. Brambell, F.R.S., for reading and correcting the manuscript.

different components at a very early stage of development when the regulative capacities are still able to integrate the two components into one entity.

The present paper is devoted to this particular approach—the experimental production of mouse chimeras by means of fusion of the cleaving eggs.

Among the vertebrate eggs the mammalian egg is, perhaps, the most suitable for this kind of experimentation. Total cleavage and scarcity of reserve materials are among the most important advantages. As previously shown in isolation experiments in the rat (*1*), rabbit (*2–4*), and mouse (*5*, *6*), the eggs display high regulative capacities. Even at the 4-cell stage the isolated blastomeres can still effect regulation (*3–6*). It is only after the third cleavage that the final segregation of the cytoplasm from the dorsal and ventral zones of the egg is achieved (*7*, *8*). During the first three mitotic cycles the position of the blastomeres is not yet determined, but during the fourth cycle the trophoblastic cells already differentiated—or as Dalcq calls them, "enveloping cells"—begin active movements on the surfaces of the inner mass cells and finally envelop them completely. The course of development during this early phase is subordinated to an early and quick segregation of embryonic and extraembryonic material. Because of the variable position of the plane of the first cleavage in relation to the plane of the bilateral symmetry of the egg, and of the fundamental spatial reorganization that precedes the formation of a blastocyst, there is no reason to expect any regular coincidence between the plane of the first cleavage and the plane of the bilateral symmetry of the embryo. These peculiarities of early mammalian development, as briefly outlined, are of great importance for the interpretation of the experimental results and should be taken into account before the generalizations inferred from developmental phenomena in other vertebrates are applied to mammals.

The experimentally produced chimeric animals resemble to some extent the mosaic individuals arising either spontaneously or after experimental treatment. Mosaics do not represent, however, a homogeneous group, and different mechanisms, such as somatic gene mutations, chromosomal aberrations, or abnormal segregation of chromosomes during cleavage, are responsible for their origin. Moreover, the size of the changed sector varies greatly and depends on the time when the causative factor makes its appearance during embryogenesis. The rarity and diversity of spontaneous cases and the difficulty of inducing a desired change in the desired place and at the desired time severely limit the possibility of experimentation.

The first attempt to fuse mammalian eggs was by Nicholas and Hall (*1*), who transferred pairs of naked, slightly adhering, 1-cell rat eggs. One embryo developed till term, but since the effectiveness of the union achieved had not been checked the result cannot be considered conclusive.

The work presented here must be regarded as a beginning of research in this field. Many fundamental problems remain unsolved and hypotheses must often suffice where factual evidence is lacking. Special emphasis is given to the means and criteria by which the degree of chimerism can be estimated.

MATERIALS AND METHODS

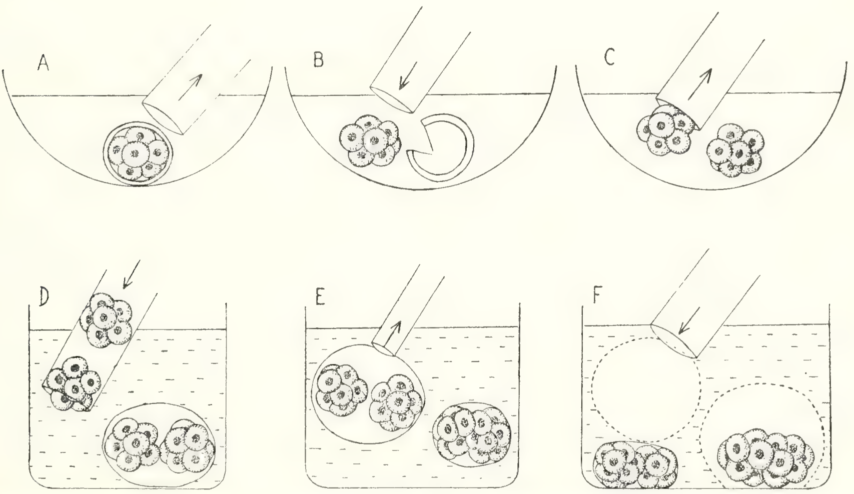
A basic requirement is that the eggs, after being fused, must be kept *in vitro* until formation of the blastocyst. The conditions of culture should permit observation at any time during the culture phase. Only when a complete integration of the two eggs into one blastocyst, which is itself morphologically completely normal, has taken place can it be used for further work. Text-figure 1 shows the steps in the fusion of eggs, which are summarized as follows [*see* (*9*) for details].

1) Eggs used throughout this work were at the 8-cell stage and were from spontaneous ovulations or from hormonally induced superovulations.

2) To remove the zona pellucida, the egg was rapidly sucked into a micropipette of an internal diameter of about 65 μ (*A*) and immediately expelled (*B*). This procedure caused rupture of the zona pellucida and allowed the naked egg to escape. The method was very effective and any damage to the blastomeres could be easily detected and the egg discarded from further experiment.

3) To fuse the eggs, two naked eggs were placed in a pipette (*C*) and together with a small drop of medium deposited in liquid paraffin (*D*). With another pipette the fluid was almost completely removed from the drop, thus squeezing the two eggs together [(*E*) and fig. 1]. Such treatment assured uniform pressure from all sides.

4) The small drops containing the squeezed eggs were placed on the bottom of a dish and left there for 5 to 30 minutes. Subsequently, a drop of culture medium was placed over each of the small drops. The latter were immediately incorporated into big drops (*F*). Though no further squeezing was applied, the eggs remained adhering together. From this time on, the eggs were not exposed to any disturbances from outside.



TEXT-FIGURE 1.—Scheme of successive steps of manipulations required for fusion of eggs. *See* text for detailed description.

5) The culture medium was based on that of Whitten (*10*, *11*) and consisted of Krebs-Ringer bicarbonate with glucose, bovine plasma albumin, streptomycin, penicillin salt, and phenol red. The duration of culture was of 24 or 36 to 40 hours. During this time, the eggs developed into blastocysts and could be transplanted to recipient mice.

Randomly bred strain LAB Grey females were used throughout this experiment. The eggs were fertilized either by LAB Grey or A_2G sperms. The hybrid between these two strains is agouti with black eyes, whereas LAB Grey animals are grey and have pink eyes. The eggs were united in the following three combinations: 1) both of pure LAB Grey origin, 2) both of hybrid origin, and 3) one pure LAB Grey egg and one hybrid egg.

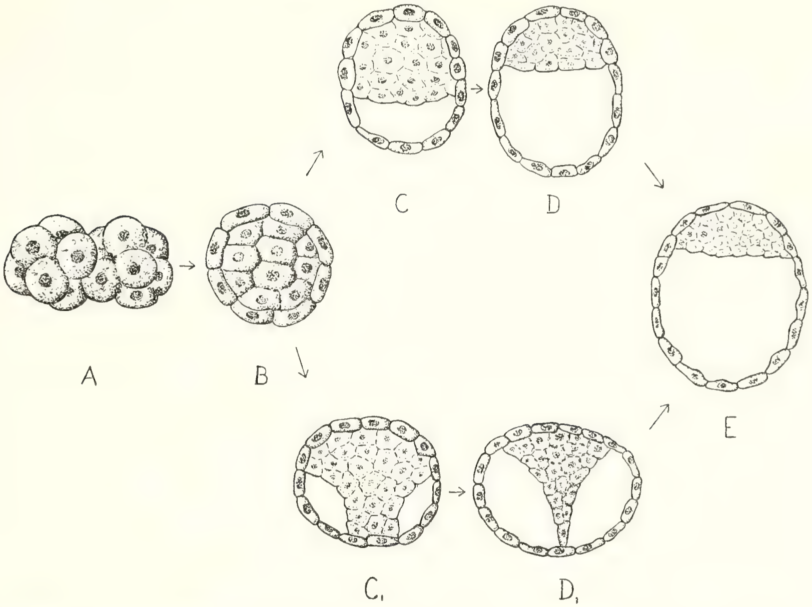
OBSERVATIONS ON DEVELOPMENT OF CHIMERAS

Culture Phase

At the beginning of culture the eggs, though adhering together, represent two distinct entities. It is during culture, when no mechanical squeezing is applied, that the boundary between the eggs gradually disappears and real union takes place. After 12 to 16 hours, each pair has rounded up into a single morula. After 24 hours, nearly all have developed into early blastocysts. At the end of a 36-hour period of culture the blastocysts are very big and swollen. The difference in size between the double and control single blastocysts is unmistakable (figs. 2 and 3). Since the eggs are cultured in shallow dishes under liquid paraffin, it is possible to observe the process of fusion and to see whether all the blastomeres have been incorporated. Any degenerate or excluded cells can be easily spotted. Usually a single blastocele appears from the very beginning. Sometimes, however, two cavities appear, separated by the fused inner cell masses (text-fig. 2). The two cavities invariably join together and blastocysts formed in this way cannot be distinguished from those developing in a normal way.

The 8-cell stage is the latest one convenient for fusing the eggs. Though older eggs adhere after squeezing, they later almost invariably separate completely to form two blastocysts. It is evident that at the 8-cell stage some special conditions must exist which enable the incorporation of the two eggs into one entity. It should be recalled that the stage at which the eggs lie in close approximation coincides with the period of movements of enveloping cells. Under the conditions of this experiment, these movements are most probably influenced by the presence of two groups of inner mass cells. The enveloping cells tend to overgrow the two inner masses together, leaving free from the beginning or retreating afterwards from the surface between them. Once the envelopment is completely, or nearly completely, accomplished, the egg represents a "closed system" and the reorganization can no longer take place.

The use of the 8-cell stage is of practical significance since it is much easier to culture than the earlier stages. Moreover, it seems likely that



Text-figure 2.—Scheme of two possible ways of development of fused eggs. (*A*) Two eggs in close approximation at the beginning of culture. (*B*) Completely rounded up morula with no trace of dual origin. In most eggs, the development proceeded according to a normal scheme (*C*, *D*, *E*). Occasionally, two separate blastocelic cavities appeared (C_1) but, gradually, they joined together (D_1). This peculiar way of development did not affect, however, the ultimate structure of the blastocyst, which looks completely normal (*E*).

the earlier stages would be unsuitable for this purpose. In 2- and 4-cell eggs, the blastomeres are only loosely connected with each other. Since squeezing cannot be applied for a long time, the whole eggs, or even the separate blastomeres, would presumably fall apart shortly after they were released from a small drop.

Development *In Vivo* After Transplantation

Of 153 double blastocysts transferred to 39 recipients, 38 embryos (including two pairs of identical twins) developed (*9*).

Autopsies were performed on the 10th, 12th, and 14th day. After publication of this work (*9*), another 6 embryos were obtained from recipients killed on the 5th, 6th, and 7th day. Regulation of the size to a normal one occurs very early, since from the 6th day onward the embryos fall within the normal range of variation in size. This regulation must take place during the short period around the time of implantation. All chimeric embryos were quite normal and no morphological abnormalities could be detected.

Birth and Development After Birth

A number of pregnant females were left until term. Some of them were allowed to litter naturally, and others were killed on the 19th day. The mice were removed by cesarian section and placed with foster mothers. Of 18 mice, 16 died or were partially eaten by their mothers during the first 3 days—most of them immediately after birth or during the first 24 hours. Two animals that survived were fertile males. One was of a pure LAB Grey origin, the other developed from LAB Grey and hybrid fused eggs. The first male attained sexual maturity after 6 weeks, *i.e.*, in normal time, and lived for 6 months. The second male was born in a litter of 4. One was eaten immediately after being placed with a foster mother, which, however, nursed the 3 others. During the first 3 days these mice, though fed, did not gain in weight. In fact the reverse happened, their weight decreased and they visibly shrank. After 3 days of life, 1 animal weighing 0.72 g died and the second weighing 0.82 g was killed. These figures are far below the average weight at birth. The third animal recovered from this crisis, but it grew very slowly. The difference in weight between this male and its foster mates persisted for at least 6 months. On the 58th day of its life, this male was paired with normal LAB Grey females, but the first mating did not take place until 2 months later when it was 4 months old.

The high mortality among chimeras is puzzling and cannot, at the moment, be explained.

METHODS AND CRITERIA OF DEFINING THE DEGREE OF CHIMERISM

The complete union of two eggs into one entity, as seen *in vitro,* is sufficient proof that at the time of transfer the cells of both components are present in the blastocyst. However, even at this stage the distribution of cells in the trophoblast and the inner cell mass is unknown. While the degree of intermingling of these cells in the trophoblast is of no great importance, as far as the composition of the embryo proper is concerned, their distribution in the inner cell mass is most significant.

One of the most important problems is to define the fate of both components in the developing embryo. In other words, what is the contribution of each of the two components to the separate systems or parts of the body of the chimeras and what constant features can be noticed?

Before considering this problem in detail two points should be emphasized. In the mouse, the initially flat, inner cell mass develops into the egg cylinder, which in turn differentiates into embryonic and extraembryonic parts. The embryo proper develops from the embryonic part and so the composition of this part only will be reflected in, and is of significance for, the composition of the chimera. Two qualifications can be made: 1) In rodents, primitive endodermal cells take their origin from the enveloping cells (*12–14*). Those endodermal cells that cover the ectodermal embryonic part of the egg cylinder contribute to the embryo proper also. 2) The blood islands are formed in the splanchnopleure of the yolk sac and, as far as the blood is concerned, the extraembryonic elements contribute, though in a special sense, to the composition of the embryo. However, the fact remains that all derivatives of the ectoderm and of at least the greatest part of the mesoderm can be traced back to the primitive ectoderm of the embryonic part of the egg cylinder. The composition of this territory at this early stage is, therefore, decisive in the composition of the overwhelming bulk of the embryonic body.

The ultimate degree of participation of both components in the chimera is dependent also on the rate of divisions of the two kinds of cells. Differing rates of divisions, especially in the early stages, could profoundly influence their ultimate share in the whole body.

What are the possible methods of tracing the lineage of cells from each of the two components and of evaluating their participation in the body of chimeras?

1) One egg could be labeled with a radioactive tracer before fusion. This would be a most precise method but limited to a rather short period due to "dilution" of the tracer. If the period when the method is still reliable covered the differentiation of the embryonic part of the egg cylinder, valuable information could be gained.

2) One component with a cytological marker could be used. Such a marker, a translocation obtained by Carter *et al.* (*15*), has been successfully used to trace donor spleen cells in blood chimeras (*16, 17*). The inconvenience of all cytological markers based on chromosomal morphology lies in their restriction to the mitotic stages only.

3) The impossibility of applying the test for Barr's sex chromatin in interphase nuclei to the mouse is unfortunate. Though such a test could be applied only to male/female genetical chimeras, it would be of special significance just in these particular cases. As a substitute, a method based on the differences in morphology of sex chromosomes can be suggested. Stich and Hsu (*18*) have shown in the mouse that it is possible to define the genetic sex of the cells by studying the metaphasic chromosomes. This method has been applied in studies of chick-embryo blood chimeras (*19*).

4) The last method is based on using for fusion eggs differing in the factor for pigmentation. I shall discuss this problem in detail because the method has already been employed and some results can be presented. In one series of experiments eggs of pure LAB Grey origin were united with hybrid eggs, resulting from a cross between LAB Grey and A_2G strains. LAB Grey animals have grey fur and pink eyes—they form melanin but in extremely low concentration. Hybrids are of agouti type with black eyes. Henceforth the words "light" and "dark" will be used to denote each component.

The working hypothesis was that if pigmentation were revealed in some form, in all or at least in most cases, this could be regarded as indirect evidence that the "light" component was also contributing to these individuals. The series consisted of four 12-day embryos, including one pair of identical twins, two 14-day embryos, and 10 young.

In the hybrid embryos pigment can be found only in the retina of the eye. In newborn hybrid young, pigment is present in the cells of the outer layer of the retina and in the dendritic melanocytes scattered in some structures of the uveal tract and in some regions of the skin. Since all but one of the chimeric young died shortly after birth, histological examination of the skin had to be carried out as the only possible means of defining coat pigmentation.

The origin of cells capable of melanin formation is twofold. The pigmented outer layer of the retina is a part of the optic vesicle and the pigment is produced *in situ*. Pigment begins to be deposited here on the 12th day of gestation. Pigmentation of the skin and hair is, on the other hand, caused by melanocytes which originate from the neural crest and migrate afterward to their ultimate locations (*20*). Melanocytes present in the choroid, nictitating membrane, harderian gland, and iris are presumably of similar origin. These facts must be borne in mind when the pigment-forming cells are used as tracers of the presence and distribution of the genetically tagged component.

Of 16 embryos and young obtained, 15 had pigmented eyes (*9*). Since this work was published, the eyes have been examined histologically (*21*). In 7 of 9 available young, the pigment-forming layer of the retina was mosaic in character with alternating pigmented and unpigmented areas (fig. 4). In 1 animal the retina was completely pigmented and in the other no pigment could be found. In mosaic eyes the ratio between light and dark areas varied from individual to individual and to a less extent even between the left and right eye of the same animal.

As mentioned, the outer layer of the retina develops from a part of the optic vesicle. Its history can be traced back first to the lateral wall of the forebrain and then to a part of the head territory of the neural plate, differentiating from the anterior region of the ectoderm. All cells in the retina can be considered as descendants of a small number of cells in the primitive optic vesicle. No other cells contribute to the retina during ontogeny. If this structure, which can be considered a fortuitous sample of the cell population, shows a chimeric constitution with such a high regularity, it can be postulated that this high degree of heterogeneity prevails also in other parts of the body or at least in its anterior region.

In the eye, besides the pigmented cells of the retina, typical pigmented melanocytes can be found in the iris, choroid, nictitating membrane, and harderian gland. In the rat, according to Bartelmez (*22*), there is a proliferation of the neural-crest cells from the forebrain and subsequently from the optic vesicle itself. According to his supposition, this may be where the predecessors of melanocytes populating these different structures of the uveal tract originate. In all but 1, an animal with no pigment in the retina, melanocytes were found in the choroid, iris, and harderian gland of the experimental animals. The number of cells showing pigment synthesis seems lower than in control animals, but accurate counts have not yet been made. If the supposition advanced by Bartelmez (*22*) proved true, the lower concentration of melanocytes in these structures of chimeras would be quite understandable.

Pigmentation of the skin and hair does not provide clear evidence for chimerism in particular regions of the body. The evaluation of observations is difficult because of the following facts.

1) The pigmented cells in the skin, as "immigrants" in this territory, characterize the genotype of the population of the neural-crest cells rather than the genotype of the tissue at the place of their final localization.

2) The route of migration of melanoblasts is not well known. It is not clear whether they migrate from the place of their origin only laterally or in a longitudinal direction as well. Ris made the only attempt, so far, to answer this question (*23*).

He cauterized a part of the neural tube—along the length of a few somites—of the chick embryo before the proliferation of the neural-crest cells, and ascertained that, after hatching, the skin of the neighboring regions was normally pigmented. This finding suggests a longitudinal migration. Such an experiment creates, however, a special situation: The part of the skin adjacent to the destroyed region of the neural tube is melanoblasts—free and open to migration from the surrounding areas. This does not happen in normal development.

Though it seems most probable that the melanoblasts populate a radially diverging sector, there is no proof, and it is unlikely that they migrate far from the level of their origin. I am inclined to think that the character and "density" of the melanocyte population in a given area of the surface of the body corresponds to the composition of the neural crest at approximately the same level.

With these considerations in mind, a search was made for melanocytes in various parts of the skin (*17*). In all but one pink-eyed animal dendritic melanocytes were detected in the area between the genital papilla and anus. This strongly suggests the presence of cells derived from the dark component in the posterior region of the neural tube. When the pigmentation of skin and hair is used as a criterion of chimerism, the presence of the "light" component cannot be proved until a general dilution or spotting in the coat of older animals is observed, or a significantly lower concentration of pigmented cells, in comparison to control animals, is confirmed in the newborn.

From these remarks it follows that a direct and strict quantitative estimate of the degree of chimerism can be carried out on the retina only. The validity of such an estimate is, however, restricted to this particular area.

All the methods, so far discussed, are based on the differing proprieties of both components which express themselves at the cellular level.

Among all systems of the animal, the reproductive system is the only one in which the consequences of a chimeric constitution can be detected at the morphological level. This holds true, however, for the female/male genetical chimeras only, which, theoretically, should represent 50 percent of all cases. Both females and males are expected to occur in 25 percent of cases.

The postnatal material hitherto collected consisted of 16 animals (*9*). Histological analysis of the genital apparatuses (*24*) confirmed the previously obtained estimate of the sex ratio based on macroscopic examination. The two young which were alive at that time, and therefore could not be strictly classified were subsequently identified as normal males since both were fertile. Among these 16 animals: 2 were females, 3 intersexes, and 11 males. The observed number of males exceeds the number theoretically expected; this excess proves significant at the 1 percent level, as shown by the chi-square value computed from a 2-cell table (Yate's correction for continuity has been applied).

The preponderance of males and the low incidence of intersexes, which should constitute the biggest group, suggest that some of the males are also sex chimeras in which the differentiation of the reproductive system followed a strict male line of development. Since there is no ground to assume a differential contribution of the cells of the male component to the genital apparatus, the original constitution of this tissue should represent all degrees of chimerism. Since this is not reflected in the ultimate structure of the reproductive system, it has to be postulated that in some cases the sexual differentiation is governed completely by the tissue derived from the male component. The experiments presented in this paper do not permit verification of this hypothesis *per se*, but some support is provided by experimental studies on the differentiation of the genital apparatus in normal development.

In considering the differentiation of the reproductive system two separate problems emerge: 1) differentiation and structure of the gonads and 2) differentiation and structure of the sex ducts and accessory glands. I shall confine myself to the first question only, as the character of gonads is decisive for the differentiation of the somatic sexual characters and is, therefore, of primary importance in the chain of inductive interactions.

In placental mammals the ahormonal type of development is feminine, *i.e.*, in the absence of gonads, either ovaries or testes, the sex ducts develop according to the female type. Presence of testicular tissue is essential for the prevention of the genetic males from differentiating as females. [For presentation of relevant investigations and discussion *see* Jost (*25*).]

For the subject under discussion only the work concerned with the role of embryonic hormones is pertinent. Macintyre (*26*) grafted, under the kidney capsule, heterosexual pairs of rat gonads from 15-day embryos. This author has shown that testes inhibit differentiation of ovaries and can, in extreme cases, induce in the ovarian territory structures resembling seminiferous tubules. Holyoke (*27*), working with the rabbit, came to similar conclusions. The masculinizing effect of testes, when cultured *in vitro* in heterosexual pairs, was less evident, but the suppression of the ovarian differentiation was clearly shown (*28*, *29*). Macintyre *et al.* (*30*) have recently demonstrated that the inhibiting action of testes is local in character and decreases with the distance between the two tissues.

In birds the dominating gonad is the ovary. In the duck, Wolff and Haffen (*31*) have shown the strong feminizing effect exerted by ovary upon testis, when undifferentiated gonads are cultured in heterosexual pairs. These experiments provided even clearer results than similar attempts in mammals. In both of these groups, the tissue of the embryonic gonad of one sex is able to control the differentiation of the gonadal elements of the opposite genetic sex, though in opposite directions.

Presumably, the difficulty in obtaining a complete sex reversal in mammalian ovaries results from the fact that the gonads used in all the experiments were already morphologically differentiated. Since the morphological differentiation is only a visible manifestation of a preexisting physiological activity, even such gonads which, at the time of extirpation for grafting or culturing, are still morphologically undifferentiated can already be conditioned to such an extent that complete sex reversal is not possible, or would require such a dose of inductive substance as the other embryonic gonad is not able to provide. In chimeric embryos of female/male genotype, the tissue derived from both components is developing as a mixed population from the time when the genital ridges are formed, and the inductive influences can be exerted from the time of their appearance. It is known that in the mouse, as in other mammals, the differentiation of testes precedes the differentiation of ovaries (*32*). The more precocious activity of the tissues of the male sex could, therefore, have a profound effect on the course of development of the whole gonad. However, since hermaphrodites do occur among chimeras, this hypothesis is not of general application and needs further elaboration.

Each of the three intersexual animals among the newborn chimeras represents a different type of gonadal pattern: ovary-ovotestis, ovotestis-ovotestis, and ovary-testis. Only the latter (fig. 5) can be described as a lateral hermaphrodite. The character of the sex ducts is dependent on the type of the adjacent gonad. Ovotestes cause persistence of both müllerian and wolffian ducts, but only one of them is well developed. A detailed description of these hermaphrodites is given elsewhere (*24*).

Spontaneous hermaphroditism is known to occur sporadically in the mouse [*see* (*24*)]. Among these cases, representing a whole range of abnormalities, some are identical with, or very similar to, those seen during the present experiment. However, while the background of spontaneous cases is obscure (presumably several different mechanisms were involved) the only reasonable way of explaining the origin of chimeric intersexes is the heterogeneous genetic constitution of their gonads. Why in some sex chimeras an intersexual gonad develops and in others, as previously suggested, a typical testis, cannot at the moment be explained. Perhaps the degree to which the cells of both components are mixed together is of some importance. According to such a hypothetical explanation, the increasing degree of heterogeneity of the territory of gonads, and generally speaking of the mesodermal layer, would be reflected in the following sequence of the types of both gonads: ovary-testis (lateral hermaphrodite), ovary or testis-ovotestis, ovotestis-ovotestis, testis-testis. Until an estimation of the genetic sex of cells contributing to the gonads of such animals is made, all these explanations are bound to remain hypothetical.

The question of the cellular constitution of the gonads is part of the wider problem of the constitution of the mesodermal layer. The embryonic mesoderm originates from the primitive ectoderm [(*33*, *34*), and others]. It can be postulated, therefore, that the chimeric character of the ectodermal structures or derivatives, such as the central nervous system, if demonstrated, can provide indirect evidence for the heterogeneous constitution of the mesodermal layer. There is no ground, however, to expect close spatial or quantitative paralellisms in both layers. In this connection it seems noteworthy that both hermaphrodites which developed from light/dark fused eggs had mosaic retinas in left and right eyes. This constitutes another indication of their chimeric constitution. What is more important, however, is that the lateral hermaphroditism of 1 of these animals is not accompanied by lateral chimerism in the central nervous system—as revealed by the constitution of retinas—and, most probably, in the ectoderm itself. It seems highly improbable that lateral hermaphrodites in mammals, for instance those described by Hollander *et al.* (*35*), represent real gynandromorphs, in the sense in which this definition has been applied to insects.

It can be concluded from the foregoing discussion that the morphology of the reproductive system does not seem to be a sensitive criterion of chimerism and that the tests operating at the cellular level should be concurrently employed.

The last problem to be presented is the estimation of the chimeric character of an animal based on the analysis of its progeny. As pointed out, both animals which survived were fertile males. One was of a pure LAB Grey origin and thus could not be tested this way. The second animal developed from LAB Grey and hybrid fused eggs. It is evident that with one hybrid component the backcrosses to parental strains could not provide clear-cut results. In control matings of normal hybrids with LAB Grey animals, 50 percent of the offspring are pink-eyed. Among 442 animals obtained from these crosses 209 had pink eyes and 233 had dark eyes. In this situation, only if the percent of LAB Grey offspring in the progeny of this chimeric animal was significantly above 50 percent could the mixed character of the population of germ cells be postulated. This did not turn out to be true. Fifteen LAB Grey females mated to the chimeric male produced 113 young—56 with pink eyes and 57 with dark eyes. There is no proof, therefore, that this animal has the germ cells derived from both components. New experiments, with two homozygous strains, must be undertaken to clarify this question.

Tests based on the analysis of progeny are of a special character since they estimate exclusively the composition of the population of germ cells and give no evidence as to the chimerism of the soma. The precursors of these cells, as shown in the mouse by Chiquoine (*36*) and verified later by other authors, originate outside the embryo—in the splanchnopleure of the yolk sac—and migrate afterward to their definitive position in the germinal ridges. Because of this, in a given region of gonads there need not be any coincidence between: 1) the genotype of the somatic cells and primordial germ cells which immigrated into this area and 2) the genetic sex of these two kinds of cells.

In addition, such tests should help check the hypothesis that some sex chimeras develop into normal males. If in gonads developing into testes there are XX-bearing germ cells and if meiosis of these cells along the male line can proceed normally, then in backcrosses to parental strains one of the two genetic types of progeny should be represented exclusively by females.

It is evident from the foregoing discussion that each of the methods for estimation of the chimeric character of an individual has its limitation and that only when they are applied together can a full characterization of an animal be reached.

In this paper emphasis has been deliberately placed on the methodology of the work. If experimental production of chimeras becomes a research tool in other fields of biology, such as developmental genetics, immunology, etc., it is important to elaborate first the suitable methods and criteria for estimation of the degree of chimerism and to know the factors governing the development of chimeric animals.

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PLATE

Plate 6

Figure 1.—Two 8-cell eggs compressed in a small drop of medium suspended in liquid paraffin.* × 400

Figure 2.—Single naked blastocyst developed from 8-cell egg cultured for 24 hours.* × 400

Figure 3.—Double blastocyst developed from two fused 8-cell eggs after 24 hours in culture * × 400

Figure 4.—Tangential section of the outer layer of retina of a newborn chimeric mouse. The animal has developed from pure LAB Grey and hybrid (LAB Grey × A_2G) fused eggs. Pigmented and nonpigmented areas are intermingled. Hemotoxylin and eosin. × 200

Figure 5.—Genital apparatus of 3-day-old chimeric mouse. Testis with epidydimis and vas deferens on *left side* of picture and ovary with oviduct and uterus on *right side*. *Note* that the seminal vesicle is developed on only the male side.* × 16

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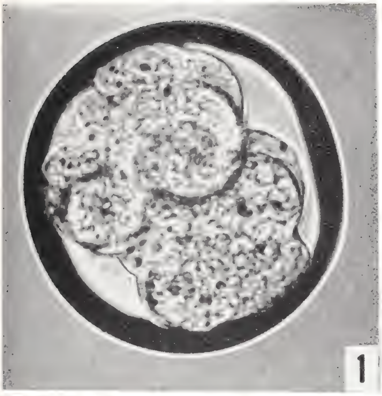
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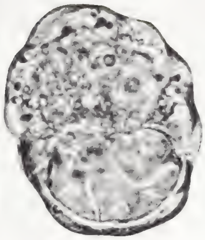
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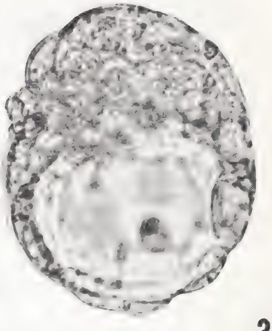
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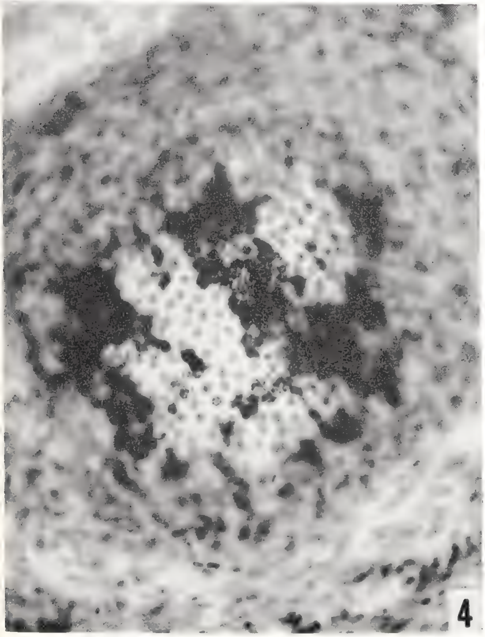
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DISCUSSION

R. B. L. GWATKIN, King Ranch Laboratory of Reproductive Physiology, University of Pennsylvania, Philadelphia, Pennsylvania

Dr. Tarkowski is to be congratulated for his pioneering experiments with fused eggs. The elegant, yet basically simple, techniques which he has developed should make it possible for us to study the interaction in the developing mammal between cells differing as to sex, karyotype, antigenic components, and even species. Before this can be accomplished, however, we must devise better methods for distinguishing the cells which derive from each contributor to the chimera.

As Dr. Tarkowski has stressed, pigmentation and morphology of the reproductive system are of only limited value in assessing the degree of chimerism; primarily because they involve only a small part of the animal. Among other possible markers, chromosomal morphology is of little value since observations are restricted to cells in metaphase. Labeling one component with tritiated thymidine would be helpful, but dilution of the isotope by cell division severely limits this method as well. Probably the best approach would be to avoid the problem of dilution by use of a cellular antigen as a marker and to locate it with a fluorescent antibody. We hope soon to explore this technique with chimeras prepared from 2 inbred mouse lines with different alleles at the *H-2* (histocompatibility) locus.

Dr. Tarkowski has said that one reason for employing 8-cell eggs for preparing chimeras is that eggs of earlier stages are difficult to cultivate. This is indeed true. So far no one has devised a medium which will regularly allow development of mouse embryos through the first three cleavage divisions. To determine whether the fallopian tube alone is sufficient to ensure normal development, experiments were carried out, in collaboration with Dr. J. D. Biggers and Dr. R. L. Brinster, with organ cultures of fallopian tubes containing fertile uncleaved eggs (*1*).

Randomly bred Swiss mice, 8 to 9 weeks old, were superovulated by the injection of 5 IU pregnant mare serum gonadotrophin followed 48 hours later by 3 IU human chorionic gonadotrophin and the introduction of fertile males. The fallopian tubes were removed 22 to 24 hours later. At this time examination always showed unsegmented ova at the top of the tube. The expected time of the first cleavage division is not for another 12 to 14 hours (*2*).

Fallopian tubes were partially uncoiled by cutting the mesenteries. They were then stretched out on circles of tea-bag paper, which in turn were placed on platforms of stainless-steel mesh (fig. 1). Contact of the medium with the wire mesh and paper was maintained by surface tension. Petri dishes were incubated in a moist atmosphere of 5 percent CO_2 in air. Details of this culture method can be seen as a demonstration at this meeting and are to be published elsewhere. The medium used was BGJ*b*, which consists of some 34 components, including amino acids, vitamins, salts, and antibiotics (*3*). Noteworthy features are the high concentration of glucose (5 mg/ml) and sodium bicarbonate (3.5 mg/ml).

Fifty-four such fallopian tubes were cultured for either 3 or 4 days and their contents examined. The results are summarized in table 1. Forty-three (80%) contained developing eggs, and 159 of the 215 eggs (74%) had developed at least into morulas, with the exception of one early blastocyst, while the tubes examined on the 4th day contained a predominant number of blastocysts. According to Lewis and Wright (*4*), morulas developed into blastocysts *in vivo* on the 3d to 4th day after fertilization, so the rate of development was unaffected by the conditions of culture. A typical living blastocyst produced in this way is shown in figure 2. This culture procedure gave similar results with an inbred line, BALB/c mated with DBA/2 males, as with the randomly bred Swiss mice (*5*).

Several blastocysts, which had developed in organ cultures of fallopian tubes, were transplanted into the uterus of inbred C57BL mice, superovulated, and mated to C57BL males 3 days previously (fig. 3). On the 17th day of pregnancy the embryos inside the uterus of the foster mothers were examined for eye color. Figure 4 shows a litter of 5 mice in which 4 have black eyes (native fetuses) and 1 is colorless (cultured

Table 1.—Development of early mouse embryos within fallopian tubes maintained in organ culture for 3 or 4 days on medium BGJ*b*

Number of days *in vitro*	Number of fallopian tubes	Number of fallopian tubes with developing eggs	Number of eggs removed	Number of morulas	Number of blastocysts
3	29	22	109	75	1 (early)
4	25	21	106	14	69

fetus). Although this result is very preliminary, it suggests strongly that the conditions of culture have little adverse effect on the early embryos. Further studies on this problem are in progress.

These results show clearly that the fallopian tube alone, in isolation from the rest of the body, can meet all the needs of the free-living phase of embryonic life. If a hormone is required, then the amount in the fallopian tube at the time of explantation is sufficient for the 4-day period of development *in vitro*. The next phase of this indirect approach to the nutrition of mouse ova will be to culture 1-cell eggs in microdroplets of fallopian-tube fluid.

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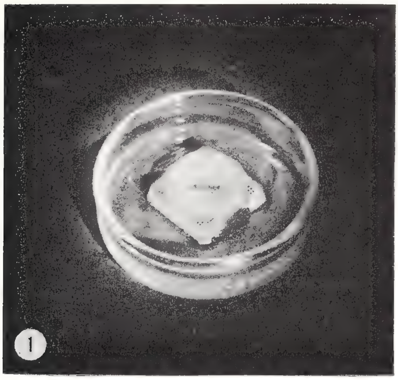
Plate 7

Figure 1.—Culture dish, 6 cm diameter, showing an uncoiled fallopian tube resting on a disc of tea-bag paper, which in turn rests on a platform of stainless-steel mesh.

Figure 2.—Living mouse blastocyst produced in 4 days inside an organ culture of fallopian tube. Phase contrast. × 720

Figure 3.—Introducing cultured embryos into exposed uterus of a C57BL foster mother. Glass micropipette is passed through hole made by the hypodermic needle.

Figure 4.—Seventeen-day-old litter from uterus of a C57BL foster mother, containing 4 native embryos (pigmented eyes) and 1 cultured embryo (unpigmented eyes).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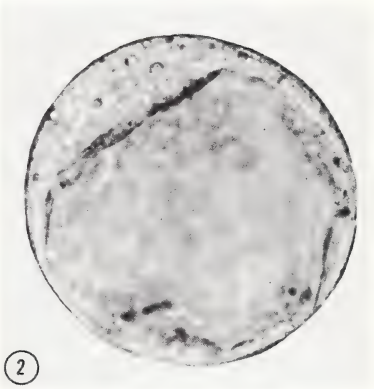
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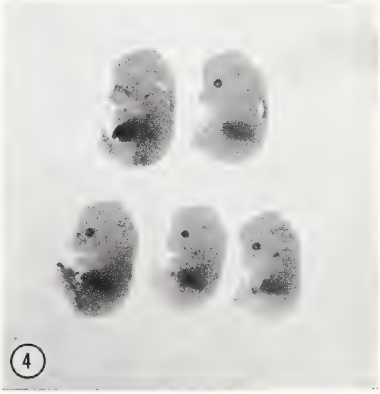
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STUDIES OF DEVELOPMENT IN ORGAN CULTURE: SUMMARY, CORRELATION, AND SPECULATION [1]

HONOR B. FELL,[2] Strangeways Research Laboratory, Cambridge, England

HISTORY

It is very gratifying to a Briton to see the growing interest in the organ-culture technique, as exemplified by the large attendance at this symposium, because organ culture originated in England. Perhaps it would be appropriate on this occasion to sketch briefly the history of this branch of tissue culture.

In 1914, D. Thomson (*1*, *2*) published a paper in the *Proceedings of the Royal Society of Medicine,* in which he recorded the important observation that isolated parts of a young chick embryo, such as the toes, feather rudiments, and lens, enlarged as a whole when cultivated *in vitro*. He applied the terms "controlled" to this type of growth and "uncontrolled" to the more familiar outwandering of cells from a cut surface. Thomson did not examine his explants histologically, nor did he pursue the study of "controlled" growth any further.

Eight years later, the progressive differentiation of fragments of early embryos was described by Chlopin (*3*) and Fischer (*4*), and in 1925 by Maximow (*5*) who referred to this type of growth as "organotypic."

In 1923, as a very new Ph.D., I joined what was then the Cambridge Research Hospital and is now the Strangeways Research Laboratory, as Dr. Strangeways' research assistant. Soon after my arrival, the Chief showed me first a little piece of embryonic intestine that he was growing on the surface of a plasma:embryo extract clot in a small centrifuge tube, and then a histological section of a fragment of articular cartilage that he had cultivated in the same way. Being a zoologist with an embryological bias, I suggested to Dr. Strangeways that it would be interesting to see whether undifferentiated organ rudiments could develop *in vitro* if they were completely isolated from the tissue with which they were normally associated. In 1926 we published experiments in which we were able to show that the undifferentiated mesoderm from the limb-bud of a 3-day embryonic chick would form cartilage in culture (*6*) and that the isolated eye rudiment from embryos of the same age would form a lens and all the characteristic layers of the retina when explanted under the same conditions (7).

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[1] Presented at The 13th Annual Meeting of the Tissue Culture Association, Washington, D.C., May 29–31, 1962.

[2] Royal Society Foulerton Research Fellow.

At the end of that year (1926), Strangeways died, but experiments with organ cultures were actively continued. For some years ours was the only institution to practice the technique, the other tissue-culture laboratories being concerned solely with cell culture. Then in 1928 an enterprising young Dutchman visited the Strangeways Laboratory, and on his return to Holland began to work with organ culture with outstanding success; his name was Peter Gaillard. We had to wait a long time before any other research center became interested in the subject.

PRESENT POSITION

But during the past decade the situation has completely changed, and we organ culturists now find ourselves quite socially acceptible among cell biologists. How has this come about? I think that there are three main reasons for the sudden popularity of the technique.

In the first place it is now generally recognized that cell cultures are unsuitable for many types of research that require a tissue-culture system. This is because unorganized cells, especially those of long established strains, differ greatly from their prototypes in a normal differentiated tissue *in vivo*; many of their physiological and morphological characteristics may be quite atypical, and they are unable to reproduce some of the specific reactions to chemical and other stimuli that are manifested by differentiated cells, because they have lost the necessary machinery for the response. Nevertheless, cell cultures will always remain an invaluable tool for virology, cancer research, and the study of growth processes.

In the second place, the recent development of microchemical methods enables us to do much more sophisticated experiments with organ cultures than was previously possible. For example, formerly we could study only the morphological changes caused by experimental factors. We could tell whether a given agent had a direct action on the tissue, which was something, but if we wished to investigate the physiological significance of the structural alterations produced, we soon found ourselves up against a blank wall. But refined biochemical techniques have opened a door in this wall, and now we can pursue our problems as far as our wits will take us.

Thirdly, Moscona, Grobstein, and Etienne Wolff's school have demonstrated, in a dramatic way, the enormous possibilities of organ culture as a means for studying intercellular relationships. The fascinating results obtained by these workers have stimulated great interest and activity in this application of the technique and I think in the subject as a whole.

Having indicated something of the past history and present-day background of organ culture, I will now turn to the 4 papers that we have heard this morning, and consider them from both the scientific and technical standpoint, as examples of how organ culture may be applied to developmental problems.

J. D. BIGGERS

For some years Dr. Biggers has been engaged in making a precise quantitative analysis of the interaction in organ culture, between cartilaginous limb-bone rudiments and their nutrient medium. He has told us that this system becomes unstable after 5 days. His criterion of incipient degeneration is a decline in the ratio of dry weight to wet weight in the explants. I should have liked to hear what happens to this ratio during the normal development of the limb cartilage *in vivo*, before accepting the decline in the ratio as conclusive evidence of diminishing viability. However, from my own experience I am sure that his conclusion that the rudiments become increasingly unhealthy after 5 days in chemically defined medium is correct, which means that this is the outside time limit for experiments with such cultures. Dr. Biggers has set himself the important task of trying to find out what goes wrong with the cultures after this stage, with the idea that an accurate diagnosis of their ailments might lead to the prevention of their decline and to the consequent prolongation of the experimental period.

I was much interested in Dr. Biggers' observation that the hydration produced by a lathyritic agent occurs only in cultures that contain living cells, and is entirely suppressed if the explants are exposed to a lethal dose of this compound. With a sublethal concentration, the explants become wetter because dry matter is lost. I am sure he is right in suggesting that the breakdown of the matrix is due to the release of enzymes from the injured cells. That this effect is not produced if the cartilage is killed implies that a lethal dose arrests the synthesis of the enzymes whereas the sublethal dose does not. It may be that the decline of organized explants during prolonged cultivation in normal medium is due to a progressive imbalance of their synthetic processes, some syntheses progressing almost normally, others being excessive, and others again being wholly or partially inhibited by the environmental conditions, until the delicate machinery of the tissue is thrown completely out of gear.

Unlike cell cultures, organ cultures are essentially for short-term experiments. There is seldom much point in trying to maintain them for long periods, because the explants usually react in a few days or weeks to such chemical agents as hormones, vitamins, and carcinogens, and most of the synthetic processes of differentiation are accomplished in a similar time. It is just as well that the experiments are of short duration, because they are very laborious; this is due mainly to the fact that they must be correlated with a careful histological study of the explants—I cannot emphasize the importance of this too strongly.

In my view, organ culture is to be regarded as an extension, not of cell culture, but of what in our Laboratory is termed "chemists' *in vitro*" by which we mean the study of fresh tissue in Krebs-Ringer or some such medium. Experiments with "chemists' *in vitro*" cannot be carried for more than a few hours, because after this stage the tissue is moribund and any results that may be obtained are suspect. This sets rather narrow limits to the physiological processes that can be studied by this simple method.

Organ culture enables the experimental period to be prolonged for several days and sometimes for a few weeks. At one time, for the biochemist this advantage was far outweighed by the difficulty of making analytical and metabolic observations on the very small quantities of tissue obtainable from organ cultures. As I mentioned earlier, however, modern microchemistry has removed this difficulty, and, as I know from our own experience at the Strangeways Laboratory, many biological processes can now be examined at the biochemical level in differentiated tissues isolated *in vitro* that formerly would not have been open to this approach.

Whether an experiment is to last for hours, days, or weeks, however, we must be sure that during the operative period, the tissue is in a healthy, normal condition. I think that Dr. Biggers is to be warmly commended for his determined efforts to define the factors that eventually impair the relationship between the explant and its environment, and thus render the tissue unsuitable for experimental purposes.

I should like to make a few remarks about the watch-glass technique which Dr. Biggers has used throughout this work.

This method was specially designed for the cultivation of *large* explants for which, on the whole, it gives very satisfactory results. The original simple type of watch-glass culture (*8*) consists of a watch glass enclosed in a petri dish carpeted with cotton wool saturated with distilled water; the medium is placed in the watch glass and the explants are grown on the surface of the medium. There are many modifications of this technique. For example Trowell (*9*) developed an excellent form of the watch-glass method whereby many types of adult tissue can be maintained in a healthy state for a week or more in a simple chemically defined medium; he was the first to show that some organized tissues thrive much better in an atmosphere of 95 percent oxygen plus 5 percent carbon dioxide than they do in air. There are various versions of his technique.

The watch-glass method is not suitable for very small explants. It is a common experience that there is an optimal relationship between the volume of the explant and that of the medium; I suspect that when the relative volume of the medium is too great, valuable metabolic products rapidly diffuse away from the tissue. I have often heard it suggested that the ideal arrangement would be a continuous flow of medium, but I am doubtful about the desirability of this.

I am often puzzled by the apparent reluctance of many people to use the quite simple methods that have been developed especially for the cultivation of organized tissue; instead they prefer to use roller tubes or flasks. I am not going to generalize about this matter, since it may well be that some types of differentiated tissue thrive equally well in vessels designed for cell cultures. I can say, however, that on several occasions I have seen photographs of histological preparations of organs grown in flasks and roller tubes, in which the explants appeared to be greatly inferior to the same tissue cultivated by standard organ-culture techniques.

Dr. Biggers maintained his explants entirely in chemically defined medium which has obvious advantages for many types of experiment; for other purposes, even for certain biochemical studies, natural medium on which most explants grow better is more suitable.

Sometimes it is possible to make the best of both worlds by transferring the explants to chemically defined medium for 24 hours or less, after previous cultivation in natural medium. Recently Mr. Dingle and I (unpublished) have used this procedure very profitably to investigate the effect of vitamin A on the proteolytic activity of limb-bone rudiments. Chemically defined medium, being itself free of enzymes, is far more suitable for estimation of the enzyme activity of explants than natural medium, but the explants grow and also respond much better to vitamin A in natural medium.

R. AUERBACH

Dr. Auerbach has used organ culture to disentangle an interaction between two tissues that normally are widely separated in space.

He has told us how previous work suggested that the thymus represents the major primordium of the mammalian immunological system, and how he was led to the idea that there is a synergistic action between the thymus and the spleen. The experiments he has described today have shown that when the thymus of a fetal mouse is cultivated by itself, it forms merely dispersed lymphoid cells subdivided by a little connective tissue and that the lymphoid structure is lost after 2 or 3 weeks. Spleen alone fails to become lymphoid, but when the two organs are explanted together they produce a single complex organ with a lymphoid follicular structure which is preserved for 5 weeks in culture.

I think the evidence strongly suggests that in the body, the spleen is colonized by lymphoid cells derived from the thymus, but it is less clear how the thymus might be affected by the spleen. If the spleen has a beneficial action on the thymus *in vivo*, it must be mediated through the blood stream, and it would be interesting to know whether in culture its effect on the explanted thymus could be transmitted through a Millipore membrane. I am sure Dr. Auerbach has considered this possibility.

J. D. GROVER

Dr. Grover's work, unlike that of Dr. Auerbach, concerns an interaction between two cell types that normally are intimately combined in the same organ. He has dissociated the lung rudiments of chick embryos of different ages into cell suspensions, and, using a very simple but efficient technique, has studied their reaggregation and reorganization under various experimental conditions. He has found that the readiness with which the lung is dissociated and the effectiveness with which the cells reaggregate decline with embryonic age. Recently Moscona (*10*) has shown that in dissociated retinal cells also the capacity for reaggregation diminishes with age, so this may prove to be a general characteristic of embryonic tissues.

It is interesting that there should be an optimal embryonic age for histological reorganization. Dr. Grover obtained the best reorganization in reaggregates from 12-day embryonic lungs. That the capacity for differentiation should decline sharply in cells from older embryos is perhaps to be expected, but personally I was surprised that the cells of 7-day lungs, which reaggregate so readily, should differentiate so poorly.

The dependence of epithelia on association with mesenchyme, of which Dr. Grover has given us an interesting example, suggests one reason for the cell culturist's experience that certain cell types either cannot survive in pure culture or are grossly abnormal by comparison with their prototypes in differentiated tissue. We have seen that an aggregate formed entirely of the epithelial cells of a lung rudiment fails to differentiate either cytologically or histologically, an observation that agrees with Grobstein's finding that the isolated epithelium of the salivary-gland primordium is incapable of development. Some epithelia rapidly degenerate in the absence of connective tissue; McLoughlin (*11*) found this to be true of the embryonic epidermis of the chick.

I agree with Dr. Grover that the interaction between the lung epithelium and the mesenchyme with which it is normally associated should not be regarded as an induction. In such a system the mesenchymal elements exert merely a "permissive action" on the epithelium; by this I mean that the epithelium can only achieve the histological destiny for which it was determined, with the aid of the stromal cells, and without this aid it either remains undifferentiated or dies.

It is clear that mesenchymal cells must provide epithelium with materials that it requires for its growth and differentiation. That some of these substances must be of a very specific character is shown by experiments like those of McLoughlin (*11*) who combined embryonic chick epidermis with mesenchyme from various organs. She found that gizzard mesenchyme prevented the epidermis from keratinizing and caused it to secrete mucus; in contact with heart myoblasts the epidermis failed to keratinize and spread into an extremely thin sheet of flattened cells resembling endocardinal endothelium, but in contact with heart fibroblasts it keratinized to an abnormal degree.

Since Moscona made his original experiments in our Laboratory in 1952, much information about intercellular reactions has accumulated. We know that cellular reaggregation is temperature-dependent (*12*), tissue-dependent (*13*), but oddly enough not species-dependent (*13*); Dr.

Grover's results have shown that it is also age-dependent. Of the actual cell-to-cell relationships in this reggregation and reorganization, however, we know little. It would seem that a sequence of three probably independent processes is involved: first random reaggregation of cells; then cellular redistribution in the mass, so that like cells clump together; and finally differentiation. I suspect that the first two processes will prove more susceptible to analysis than the third. Eventually we may hope to define the physicochemical properties that cause cells to stick together in a random mass and later to sort themselves out into like groups. But after this stage is complete, how does a reaggregate of kidney cells, for example, contrive to form glomeruli, collecting tubules, and all the complex apparatus of an adult kidney, as demonstrated in the beautiful experiments of Weiss and Taylor (*14*)? As these authors remark, " . . . just how, will have to occupy research workers for some time to come"—a masterly understatement.

A. R. TARKOWSKI

I come now to Dr. Tarkowski's experiments on mouse chimeras. Dr. Tarkowski has told us how he took eggs at the 8-cell stage, removed the zona pellucida, and by a delicate and ingenious technique caused pairs of eggs to fuse *in vitro.* To make sure that the fusion was effective, he kept them under observation for a further 24 to 48 hours in a simple form of culture. During this period the fused eggs developed into apparently normal blastocysts; at this stage they were transplanted into mice where their development continued, sometimes up to birth. Those individuals that survived birth, however, lost weight and nearly all died within a few days; only 2 animals partially recovered from this crisis and reached maturity. At present Dr. Tarkowski cannot offer any explanation of this high mortality among the chimeric young.

The occurrence of true intersexes among the chimeras is very interesting. So also is the finding that when the eggs of breeds with pigmented and nonpigmented eyes were combined, in the resulting chimera the pigment layer of the retina was a mosaic with alternating pigmented and nonpigmented areas.

I agree with Dr. Gwatkin that this elegant technique has possible applications to other types of problems. For example, I wonder whether this might not be an excellent approach to the vexed question of whether deoxyribonucleic acid (DNA) can act as a transforming factor in vertebrates. The eggs might be cultivated in the presence of DNA from a different genetical strain for 48 hours and then implanted into an animal.

I think that more use should be made of organ culture in combination with grafting techniques. In developmental studies there are many microsurgical procedures that, like the one described by Dr. Tarkowski, are fairly easy in an organ culture but difficult or impossible *in vivo.* On the other hand, no *in vitro* system has yet been devised that compensates a tissue for the lack of a blood supply. This is admirably demonstrated by Weiss and Taylor's experiments that I have already quoted, in which cell aggregates grafted on the chorio-allantois reached a much more advanced stage of differentiation than they achieved in organ culture.

In closing, I want to underline the importance of keeping Organ Culture's feet firmly on the ground. It should be regarded strictly as an ancillary technique to experiments and observations *in vivo.* In our Laboratory, during the course of an investigation we constantly dodge to and fro between animal experiments, organ culture, and straight biochemistry, according to the type of information that we happen to require at the moment, and we find this to be the most fruitful way of exploiting the organ-culture method.

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Session II

Studies of Function in Organ Culture

Chairman: George O. Gey, *Johns Hopkins School of Medicine, Baltimore, Maryland*

NUTRITIONAL AND OTHER FACTORS INFLUENCING THE BALANCE BETWEEN FUNCTION AND PROLIFERATION IN ORGAN CULTURE[1]

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SUMMARY

Cells in culture, whether in organized form or not, are in many respects grossly abnormal. They may respond in an unexpected way to the constituents of even relatively simple chemically defined media. Epithelial cells alone may be incapable of normal function. The functional unit may be an epithelial-stromal complex. The lack of function in proliferating cells may be due partly to a disturbance of the epithelial-stromal relationship. All these conclusions must be kept in mind when changes occurring *in vitro* are compared with those in the living animal.—NCI Monogr. No. 11: 83–94, 1963.

SOME OF the terms in the title of this paper are straightforward. *Function* can be defined as an activity proper to anything or a mode of action by which it fulfills its purpose. *Proliferation* is reproduction or multiplication of similar forms. But what do we mean by *nutrition*, particularly in this context? As Waymouth (*1*) pointed out, we must ask, Nutrition for what? Cells that are proliferating must be supplied with the raw material necessary to make new cells. Cells that have some special function must have the raw material necessary for them to produce the specific product essential for that function. Thus it is generally agreed that a tissue-culture medium must be tailored for a specific purpose and a specific tissue. Since our aim is precision we must use a chemically defined medium. The usual method of finding the essential constituents is, of course, to omit each component in turn, but Biggers and his colleagues (*2*) have pointed out a disadvantage to this technique. They assume a growth medium in which one constituent can act as an alternative for another. If either one is omitted they appear to be nonessential, yet if both are omitted there is no growth response. The implications of this, if one is concerned with testing the individual components of a complex medium, need not be stressed.

If this obstacle is not big enough we can consider our next problem: How do we measure proliferation or function? There are several methods for giving some rough guide to the degree of cell proliferation, but how does one measure function in a structure like the human finger in organ culture (fig. 1), or elsewhere, or in a gland like the prostate that has no known function?

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[1] Presented at The 13th Annual Meeting of the Tissue Culture Association, Washington, D.C., May 29–31, 1962.

While we are in this critical mood, I think I ought to point out that we do not know whether there is a balance between function and proliferation. It is possible that some cell types may exist only to proliferate. A parental or stem cell may divide to produce one cell like the parental cell and another capable of "differentiating" into a "functioning" cell. The function of the stem cell may be proliferation.

The problem is a difficult one, whether we approach it from its practical or its semantic aspects. In these circumstances I believe it best, in the the time available, to discuss some relevant points of special interest to me, particularly as they illustrate a number of pitfalls. Factors influencing function and proliferation may lie in the tissue itself or in the environment and in this sense may be nutritional.

TISSUE FACTORS INFLUENCING FUNCTION AND PROLIFERATION

The first factor I shall discuss is a direct, partly unavoidable, but little recognized result of the method itself. The tissue to be cultured must be removed from the animal and made into a form suitable for culture; that is, it must be small enough to allow free diffusion of nutrients, oxygen, and waste products of metabolism through its whole thickness. In practice this means that each culture must be not more than 0.8 mm thick and about 2 mm in its greatest diameter. Tissues from adult organs must therefore be cut or dissected and this leads to a series of regenerative changes (*3*) which I shall show you in some mouse prostate organ cultures though, of course, the changes also occur in other tissues. Tissues dissected and prepared as for culture and then fixed immediately are similar in appearance to the normal gland, except for an occasional pyknotic epithelial cell. After 24 hours' incubation, however, many cells show a peculiar degenerative change usually involving only the cells of acini at the edges of a culture. After 3 days, the epithelium shows great mitotic activity in areas similar to those that earlier showed degenerative changes. Thymidine uptake is also much greater in these areas. In some cultures the cellular regeneration is irregular and bizarre. After 7 days, mitoses are uncommon but the irregular cells persist throughout the period of culture. This is almost certainly a regenerative reaction following nonlethal trauma during dissection.

It is important to recognize the existence of these changes for two reasons: 1) They may appear to be due to the treatment applied to the culture, particularly if the whole explant is involved. 2) These regenerated cells are not normal and *will not "function"* if treated with a substance that stimulates secretion in the normal cells. The cells do not function even after proliferation has ceased.

To illustrate a second aspect of the problem I want to describe another experiment. In organ cultures the main mass of tissue keeps its normal organized form but around the edges there is an outgrowth of cells in sheets. Mitoses are often seen in these cells. This type of growth is similar to that occurring in the more usual methods of cell culture; thus one can compare differences in structure and response of cells in an organized form, and in proliferating cell sheets, in the same environment. When mouse prostate cultures are treated with a secretory stimulant—in this case testosterone—the cells in the acini secrete normally, whereas those in the disorganized sheets apparently do not (*4*). The failure of these cells to respond may be because they are in a physical state favoring growth rather than function. Another possibility is that the epithelium alone may not be able to form secretion but can function only when it is in its normal relationship to stroma and muscle. The functional unit may be the epithelial-fibromuscular complex rather than the epithelium alone. In the mouse breast, Lasfargues (*5*) has shown that epithelium alone could not form milk fat. An organized unit of epithelium, stroma, and adipose tissue was needed before normal secretion could occur. We have become so used to the idea of epithelial secretion we tend to forget that epithelium in isolation does not exist in the intact animal. We also tend to assume, largely because of morphological similarity, that the functions of connective tissue and plain muscle are the same in every organ. This is an assumption we should hesitate to make for epithelium.

The lack of function in the epithelium in the outgrowth, therefore, may not be due to the fact that the cells are proliferating but to the absence of other cells in the gland complex that are necessary for normal function. Even the complex individuals who make up our human society are dependent to some extent on their neighbors—"No man is an island entire of itself" (*6*)—so that at the cellular level it is hardly surprising to find that there may be an interdependence between the cells of a single organ.

The importance of cellular interaction in embryogenesis has been recognized for some years and has been investigated experimentally by Wolff, Grobstein and his colleagues, and others. Some of this work was reviewed by Gaillard at the 1956 Tissue Culture Conference (*7*) and more recently by McLoughlin (*8*), but little is known about the relationship between tissue cells in the adult. In an interesting review, Herrmann (*9*) refers to some work done about 20 years ago on the choroid plexus of the brain and the ciliary body of the eye. In both these structures the epithelia contained oxidative enzyme systems, while the stroma contained anaerobic dehydrogenases. In the cornea he found that the stroma probably supplied a substrate for enzymatic oxidation in the epithelium. If the epithelium was removed, a number of syntheses in the stroma were affected. There was a mutual interdependence between epithelium and stroma. Both were necessary if the structures were to function normally.

In some experiments started while I was working with Dr. George Gey at the Johns Hopkins Hospital, we began to investigate this problem in another organ—the human adult prostate. We separated the epithelium from the stroma mechanically, using a technique for which Dr. Gey was largely responsible. The isolated epithelium would not grow though morphologically it appeared normal, but there was a rapid outgrowth of epithelium if explants of epithelium and stroma were made. Similarly, in preliminary experiments, Dr. Horst Schirmer, also of Johns Hopkins, found that isolated prostatic epithelium, though it respired, could not glycolyse, whereas a tissue slice from the gland could use both these metabolic pathways. All this work, of course, is in its early stages. It is possible that these results may be due to the fact that certain essential enzymes were leached out of the isolated epithelium during separation, as has been shown to happen with other methods of preparation of cell suspensions (*10*) or to any one of a number of other causes. But these experiments, even though incomplete, together with the others I have mentioned, do show the importance of cellular interaction in normal function. It is possible that the lack of function in proliferating cells in culture may, in part at least, be due to the disturbance of the normal relationship between the different constituent cells of the tissue.

Another factor I should like to remind you of is the age of the tissue in the explants. It is widely held that tissues from old animals are more difficult to culture than those from young ones. Yet I sometimes wonder whether this is altogether true. Certainly we have found that the post-traumatic regeneration I described earlier is just as extensive in tissue from old mice—over 2 years old—as in tissue from young or adult animals. Although the capacity of old tissue to regenerate is apparently unimpaired, its ability to function is much reduced. With the prostate again as an example, testosterone will stimulate secretion *in vitro* in glands from 6-month-old mice but not in glands from 2-year-old animals (*3*).

Lastly, we must not forget that many enzymes which may be necessary for normal function are lost rapidly when tissues are explanted, as has been shown by Ebner, Hageman, and Larson (*11*) in cultures from the cow mammary gland and by my colleague Dr. O'Shea in prostate cultures.

There are of course many other tissue factors influencing function and proliferation which time does not allow me to discuss.

ENVIRONMENTAL FACTORS INFLUENCING FUNCTION AND PROLIFERATION

Many environmental factors are obviously necessary for life: the right physical conditions of temperature and *p*H, availability of oxygen and essential nutrients, and so on. These I do not propose to discuss. But life alone, even for cells, is not enough so that I must consider briefly some factors necessary for the other components of the triad: liberty and the pursuit of happiness. Cells may be born free—even this is open to doubt—but after this event their liberty is restrained by many controlling factors. Some control their growth and some their function. Many of these controlling factors are in the most commonly used nutrient solution, blood serum. When we give our explants serum, as well as a nutrient broth, we also give an unknown and variable dose of these agents. Some, like a number of hormones, have a known effect on growth or function, but the action of others is still unknown. It is for this reason, of course, that many of us have turned to the use of chemically defined media.

The medium we chose was Trowell's T8 (*12*) because it is easy to prepare. This medium consists of a group of amino acids Eagle (*13*) found necessary for the growth of mouse cells in culture, together with salts, glucose, insulin, *p*-aminobenzoic acid, thiamine, and an antibiotic (chloramphenicol). We compared the growth of mouse prostate cultures in this medium (*14*) with growth in 20 percent human serum in Hanks' saline. After 7 days, cultures in serum-containing medium retained their normal structure. The epithelium was of normal height and in its normal relationship to muscle and stroma. After 7 days' culture in T8 there was an irregular proliferation of the epithelium. This was an unexpected finding since T8 is known to be deficient in a number of essential nutrients. The only substance likely to be growth-stimulating was insulin, which has been added to tissue-culture media for many years because it was thought to stimulate the uptake of glucose. We therefore omitted insulin from T8 and found that this medium was no longer growth-stimulating, but the cultures survived for only 7 to 10 days. Thus it seems that insulin, a hormone which was not then known to have any effect on secondary sex organs, was a growth-stimulating agent for prostatic epithelium. Since for some years we had been trying to produce an experimental prostatic cancer, we thought it might be interesting to transfer these hyperplastic cultures into a nutritionally more complete environment. We therefore transferred a number of such cultures into a serum-containing medium or transplanted them back into mice. Again, to our surprise, instead of further growth being stimulated, after 7 days in the new environment the hyperplastic cells were destroyed and the structure of the gland returned to normal. There are two stages in this process: 1) a reduction in mitotic activity and 2) destruction of the hyperplastic cells. The first may be due to the removal of the stimulating agent, insulin, since it occurs if cultures are taken from T8 and put into T8 without insulin. In this case the hyperplastic cells persist, even though mitosis is diminished or absent. In cultures in serum-containing media or in transplants, these cells are destroyed, with only a single normal layer of epithelium left. The degeneration is thus selective and since the glands are restored to their normal structure it is possible that the factors concerned may be those associated with the regulation of normal organ size in growth or in repair after injury. A large part of our current research is concerned with the isolation of these factors in human serum and with technical methods for assessment of growth and function quantitatively in organ cultures.

Since T8 without insulin would not support our tissues in the style to which they had become accustomed, we next used a rather more complicated chemically defined medium, Waymouth's 752/1 (*15*). In this medium the cultures were well preserved. After 1 week the structure was normal but after 2 weeks the columnar epithelium had undergone squamous metaplasia—another unexpected finding. It is obvious that the search for a simple maintenance medium is not yet over.

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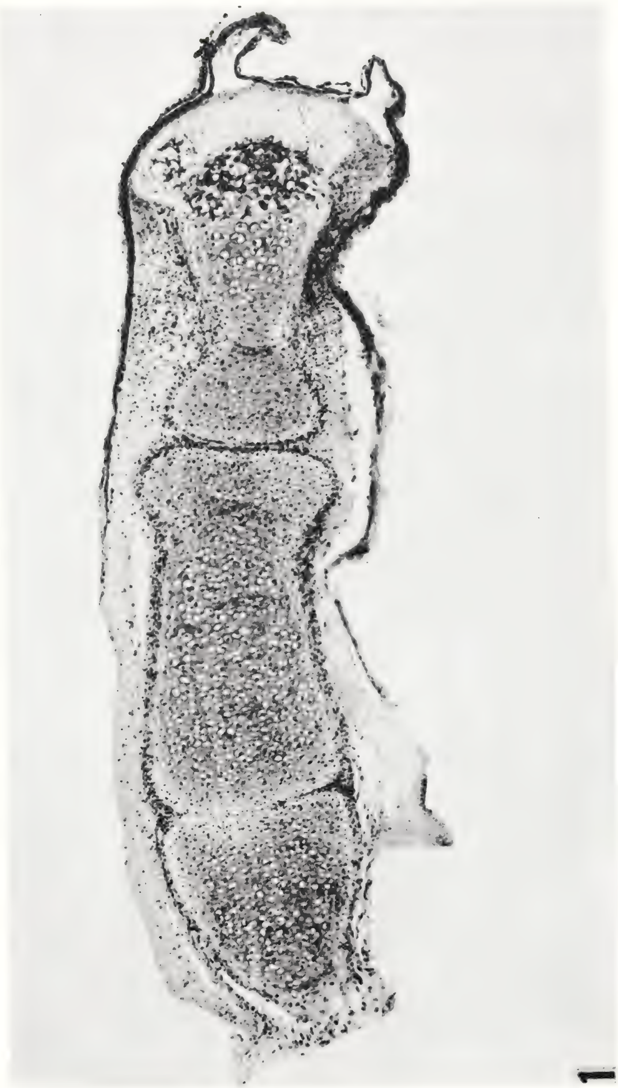
PLATE

PLATE 8

FIGURE 1.—Human embryonic finger. Twenty-four days' culture. × 70



FRANKS

DISCUSSION

An Analysis of Cell Function in Serially Propagated Cell Cultures as Compared to Studies of Function in Organ Cultures. RICHARD SCHINDLER, Pharmakologisches Institut der Universität Bern, Bern, Switzerland

When we look at the experimental tools that lend themselves to long-term studies of function, we can distinguish several systems of different complexity: 1) the intact animal, 2) isolated (and surviving) organs and tissues such as the prostate cultures described by Dr. Franks, 3) primary cultures of freshly isolated cells which, in many cases, are not capable of continuous reproduction *in vitro*, and 4) continuously multiplying cell cultures, some of which may be subjected to cloning procedures. While each of these systems has its merits, morphological criteria usually are applicable only to 1) and 2). The methods with dispersed cells, however, are particularly well suited for quantitative studies, and those systems are to be preferred in which function expresses itself as some specialized biochemical activity. Until now only few examples of exponentially multiplying cell cultures have been reported in which cell function was maintained during progressive cellular reproduction. Of these, cultures of a murine mastocytoma [strain P-815 (*1, 2*)] and of human anterior pituitary (*3*) have probably been studied most extensively, and in this discussion some of the results obtained with these systems will be summarized.

Nutritional factors.—For the cultures of human anterior pituitary, Eagle's medium (4) supplemented with undialyzed serum was used. On the other hand, the P-815 neoplastic mast cells do not multiply in semisynthetic media (containing dialyzed serum) such as that described by Eagle. This is due to two nutritional peculiarities of these cells. First, they exhibit an unusually high requirement for folic acid. In addition, media containing dialyzed serum do not support the reproduction of these cells, while undialyzed serum enables them to multiply indefinitely (*2*). Recently, we identified the growth factor which is supplied by undialyzed serum as L-serine. If this amino acid is added to the medium at a concentration of 0.1 μmoles per ml, the low-molecular components of serum are no longer required for the reproduction of the cells, and dialyzed serum may be used for the preparation of the medium.[1]

The neoplastic mast cells, obtained as ascitic suspensions from mice, begin to multiply regularly in this medium after a lag period of only 1 to 2 days. Owing to this relatively short lag period, a cell population in culture is obtained which is highly representative of the original tumor-cell population. In contrast to most other cell types, the majority of the mast cells does not attach to the glass surface of the culture flask. Other cell types in the ascitic fluid, however, become attached to the glass surface. It is thus possible to separate the mast cells from these other cell types. Similarly, epithelial-like cells were selected for subculture from the cultures of anterior pituitary to separate them from fibroblast-like cells and to obtain populations consisting exclusively of functioning cell types.

The neoplastic mast cells, under the conditions described, multiply indefinitely with generation times often as short as 10 hours, and their capacity to synthesize 5-hydroxytryptamine (5-HT), histamine, and heparin, as well as their metachromatic staining properties, are fully maintained during continued culture. It is of considerable interest that these mast cells, when grown in culture, exhibit a higher synthetic activity, as compared to cells growing as ascites tumors in mice. This difference was also observed regularly with several sublines of the P-815 mastocytoma (*5*). On the other hand, attempts to stimulate the production of 5-HT, histamine, or heparin by the addition of metabolites, such as uridine, glucosamine, pyridoxal, serine, histidine, tryptophan, or 5-hydroxytryptophan, were not successful (*5*).

Cellular factors.—By means of a selection *in vitro* a cell line called the T-line with distinctly different characteristics, as compared with those of the original cell population, was obtained (*2*). From this T-line two clonal sublines, X-1 and X-2, were developed by two consecutive cloning procedures. These three sublines showed marked differences from the original line and from each other with respect to their intracellular levels of 5-HT, histamine, and heparin (*2*, *5*). Since these differences were observed under identical conditions and were maintained with continued cellular reproduction in culture or *in vivo*, it appears that they may have a genetic basis.

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[1] These studies were supported by the Schweizerische Nationalfonds zur Förderung der wissenschaftlichen Forschung. Similar results were obtained independently by G. A. Fischer.

In summary, it is clear that these two examples of cell cultures maintaining a specialized cell function during progressive cell reproduction are too limited to warrant any general conclusions. Nevertheless, it is evident that the possibility of obtaining such cell cultures with continued cell function is not restricted to one cell type, *e.g.*, epithelial cells, or neoplastic cells alone. Futhermore, the results described demonstrate that rapid cell reproduction is fully compatible with a specialized cell function. To obtain cultures with continued function, both of the neoplastic mast cells and of anterior pituitary, a separation of the functional cell types from other cells of the original explant had to be carried out. By this procedure the possibility that the functioning cells may be overgrown by other cells during progressive culture is excluded. Thus, cell function in culture is not necessarily dependent, as in the prostate cultures, on an interaction between different cell types.

The work on the neoplastic mast cells points to the importance of nutritional factors for the culture of specialized cell types. The dependence of prostate epithelium on stroma reported by Dr. Franks seems to be another example of a special nutritional requirement which, in this case, is satisfied by the elaboration of the required nutrient by the stroma. If, as suggested, this difference is due to a deficiency in the glycolytic metabolism of the epithelium, it should be possible to substitute the nutritional effects of the stroma by supplementing the medium with the corresponding metabolites, *e.g.*, pyruvate. It is also tempting to speculate that the reported action of insulin on the prostate cultures may be related to this glycolytic deficiency of the epithelium.

Of the mast-cell neoplasm, several stable sublines have been described which exhibit quantitative differences in their synthetic activities. In this system, therefore, function appears to be a cell characteristic transmitted on a genetic basis which may be altered by mutations. On the other hand, as described by Dr. Franks, in prostate cultures the capacity of epithelial cells to carry out their function (secretion upon hormone stimulation) is easily lost under conditions of either degeneration and regeneration due to external trauma or of cell proliferation at the edge of the culture. Are these alterations leading to a loss of cell function to be considered as irreversible and possibly as reflecting a somatic mutation, or are they rather due to a change in the immediate environment of the cells?

Finally, I would like to congratulate Dr. Franks on his observations of the activity of serum in restoring the normal structure of the prostate gland. The best definition of the neoplastic state probably still is based on the fact that cancer cells evade the control mechanisms of the host, which regulate cell reproduction of normal tissues. The response of the hyperplastic prostate epithelium to serum appears to provide a beautiful model system for such a control mechanism, and this might provide the basis for an analysis of the nature and mode of action of the corresponding growth-controlling factor. Furthermore, such an analysis might, perhaps, also lead to a better understanding of the neoplastic cell.

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LONG-TERM SUPPORT OF INTACT SKIN IN ORGAN CULTURES, WITH APPLICATION TO STUDY OF VIRUS INFECTION[1, 2]

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SUMMARY

Full-thickness human fetal skin explants cultured on chicken plasma clots between 34° and 37° C remained healthy for 3 months and viable for more than 6 months. The size of cultures increased for several weeks and thereafter remained constant. The histologic appearance of uncultured fetal skin was compared with that of skin after various periods of culture. During the first 1 or 2 months after culture, explants appeared to differentiate, though the degree of maturation did not equal that in uncultured skin of an equivalent age. The epidermis increased in thickness, the basal cells became columnar, and a basement membrane and pigment appeared. While the follicles remained essentially unchanged, the ridge primordia became larger and more numerous. The dermis became thinner and the collagen more dense. New tissue appeared as an avascular, reticular, fibroblastic connective tissue. After this period the epidermis and dermis became thinner and the follicles and ridges smaller and less numerous. Inoculation with herpes simplex virus was followed by nuclear changes, giant cells, and necrosis and vesiculation in the epidermis, which closely resembled changes described in human patients, laboratory animals, and tissue culture. The results of the present study indicate that this system offers a means of producing true disease, as contrasted to infection, and of studying details of pathogenesis, virus specificity, and host-parasite relationship apart from the influence of circulation.—NCI Monogr. No. 11: 95–115, 1963.

INVESTIGATIONS OF disease agents carried out by the use of routine tissue-culture methods may furnish much information about the agents themselves, but reveal little about the pathogenesis of the diseases in the natural hosts. On the other hand, in investigations of diseases which have been made in human volunteers or in laboratory animals, certain features have been obscured or distorted by factors such as hormones and

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antibodies which are intrinsic in the circulation of the host and cannot be completely evaluated. The support of intact organs for periods of several weeks in culture may permit the production of pathologic conditions uncomplicated by circulatory factors and thus allows more complete analysis of pathogenic processes.

Matoltsy has reviewed studies of human, mammalian, and avian skin in culture (*1*). Few studies of human embryonic skin have been made (*2–4*). In a preliminary report from this laboratory, the maintenance of full-thickness human embryonic skin *in vitro* for periods up to 6 months was noted, and the suitability of organ cultures for the study of skin diseases was suggested (*5*). With use of the method described in that report, it has been possible to observe the gross and microscopic characteristics of explants after various periods of culture, in the absence and in the presence of herpes simplex infection, and to compare skin in such cultures with skin *in vivo.* In the present report these observations on a group of 641 explants are described.

MATERIALS AND METHODS

Of the 641 skin explants cultured, 122 were prepared from 6 Negro fetuses obtained in Baltimore, and 519 from 21 white fetuses shipped from Copenhagen. The estimated duration of gestation of the fetuses varied from 12 to 24 weeks, 372 explants being prepared from 16 fetuses of 16 weeks or less, and 269 from 11 fetuses of 17 weeks or more. All were cultured at 34°, 35°, or 37° C on the chicken plasma clot with chick-embryo extract medium previously described (*5*).

A total of 154 cultures from the present series was inoculated with suspensions of herpes simplex virus. From 57 of these, serial sections stained with hematoxylin and eosin were examined, and electron microscopic and virus studies not reported here were carried out on the remainder. Each culture was inoculated with 10 to 100 yolk sac LD50 of virus, as evidence of infection was not observed after smaller doses. Culture following inoculation was at 30°, 35°, or 37° C.

RESULTS

Gross Observations

Explants were considered to have survived, and to be viable, only when new tissue appeared about the edges after culture; 85 percent of the explants thus showed evidence of viability. There were no significant differences between survival at different culture temperatures or between explants obtained from fetuses of different ages (table 1).

On an average of 5 days after explantation, groups of cells extending out from the base of the explant over the surface of the clot in the form of small crescents attached to the rayon threads could be seen. Within

TABLE 1.—Proportion of 641 explants surviving on culture

Culture temperature (°C)	Age of fetuses, weeks: 12–16 Number	12–16 Percent	17–24 Number	17–24 Percent	Total Number	Total Percent
34	55/58	95	34/43	79	89/101	88
35	136/146	93	100/129	78	236/275	86
37	132/168	79	87/97	90	219/265	83
Total	323/372	87	221/269	82	544/641	85

a few more days, the groups of cells had formed masses about the edges of the originally square explant which was becoming rounded (fig. 1), and soon the explant was surrounded by an irregular zone of new tissue up to 1 mm in diameter, thicker centrally and only one cell thick peripherally, with the almost hemispherical explant covered by a white, translucent surface. Thereafter, in some cultures the zone of new tissue continued to increase until by an average of 19 days it had reached the maximum diameter possible on the rayon base, though in the majority this maximum diameter was not attained (fig. 2). Although some cultures remained unchanged in size and appearance for periods up to several months, most remained viable, as evidenced by vital staining, histologic examination, or response to inoculation with virus. The rate of growth of explants obtained from younger fetuses was perceptibly greater than that from older ones (table 2).

TABLE 2.—Proportion of 641 explants showing initial and maximum growth, and range of days on which each was attained

	Growth, weeks: 12–16 Initial	12–16 Maximum	17–24 Initial	17–24 Maximum	Total Initial	Total Maximum
Number	323/372	74/161	221/269	29/71	544/641	103/232
Percent	87	46	82	41	85	44
Range, day	3–6	8–22	4–11	19–28	3–11	8–28
Average, day	4	16	6	25	5	19

In some cultures, pigment that was not identifiable at the time of explantation appeared after several days in the form of brown granules about the dermoepidermal junction and about the hair follicles and increased slowly in amount and extent, which eventually appeared deep in the dermis and in the new growth. Occasionally rete ridges, which were not present in the uncultured skin, were observed forming and elongating to extend into the deep dermis and the new growth.

Most cultures were used for experimental purposes, but approximately 35 percent were discarded because of degeneration, mechanical distortion, contamination, or death as evidenced by the development of a dull, white, opaque appearance and by retraction of the peripheral new growth (table 3).

TABLE 3.—Disposition of 641 explants during consecutive weeks of culture

Week	Number present at beginning of each week	Used experimentally	Discarded
1	641	32	35
2	574	65	59
3	450	119	21
4	310	28	56
5	226	96	14
6	116	48	13
7	55	4	7
8	44	12	5
9	27	5	1
10	21	1	3
11	17	2	4
12	11	1	0
13–27	10	6	4
Total	641	419	222

Microscopic Observations

Before Culture

Hematoxylin- and eosin-stained sections of the uncultured human fetal skin showed no detectable degenerative changes associated with the length of the interval between death of the fetus and culture of the skin, and resembled previous descriptions of fetal skin (*6, 7*). The skin of younger fetuses (fig. 3) was covered with an epidermis of two strata: The basal-cell layer was composed of one or two layers of regular, cuboidal, dark-staining cells with large nuclei and smooth basal surfaces but with no clearly demonstrable basement membrane; the surface stratum was composed of two or three layers of large cuboidal cells having smaller, dark-staining nuclei lying at the sides of the clear, vesicular cytoplasm, and there was no flattening of surface cells or evidence of cornification.

In skin from older fetuses (fig. 4) the cells of the basal layer were more columnar and often showed a definite palisading, and a basement membrane was occasionally observed. Overlying the basal layer, the intermediate layer consisted of one to three layers of large cuboidal cells with clear or with faintly eosinophilic cytoplasm and dark, laterally displaced nuclei; in this layer the more superficial cells were flattened. Superficially, there was a layer of two or three cells which were more eosinophilic and in which the nuclei were less apparent, so that it had almost the appearance of a true stratum corneum.

Small foci of thickening and downward convexity of the basal-cell layer were observed in some of the younger specimens, in the primordia of ridges, sweat glands, or hair follicles, and most also showed the projection into the dermis of early follicles without hairs, the tip of each follicle being surrounded by a condensation of dermal cells. In the older specimens there were numerous well-developed follicles containing hairs, and early rete ridges were often present.

The dermis was much thicker than the epidermis and consisted of three zones. Outermost was a very thin layer, most apparent in younger specimens, of small dark-staining nuclei. Beneath this was a much thicker layer of larger, paler nuclei, and innermost a still thicker zone of slightly smaller and darker nuclei. A reticular type of collagen was present in all layers, but the fibers were least numerous and least dense in the deepest layer. In the older specimens the collagen was less reticular and more dense than in the younger. Blood-filled capillaries were present throughout all layers of the dermis, and in some specimens extravascular erythrocytes were also present.

After Culture

After culture, skin followed a pattern of development which was essentially the same in all cases except that those explants from older fetuses usually showed a greater degree of differentiation in all stages than did those from younger ones. The degree of differentiation observed in a culture was somewhat less than that in uncultured skin of a corresponding age, in contrast to the observations of Chlopin (*2*). In all cultures the surface of the explant soon became covered by a thick layer of loose, reticular material resembling keratin, often containing nuclei, which was at first surmounted by one or two layers of large clear cells with small nuclei of the type that originally composed the surface of the epidermis. This persisted throughout the life of the culture, became more dense after several weeks, and frequently was overlain after 3 to 6 weeks by a very thin layer of epithelium which grew upward from the edge of the explant.

Within 10 days after explantation (fig. 5) the epidermis increased to five to ten cells in thickness, the basal cells becoming columnar and parallel, with some vacuolation of the cytoplasm beneath the nucleus and a definite basement membrane, and the intermediate layer growing thicker with some flattening and loss of vacuolation of its outer cells. At about this time a lateral extension of the epidermis could be observed, usually outward and upward to form a characteristically thickened and distorted mass of epidermal cells at the border of the original explant. The dermis became thinner and more cellular and the collagen more compact. New growth appeared beneath and at the sides of the explants, in the latter case it appeared as a reticular, fibroblastic type of tissue, sharply distinguished from the original dermis, overlain by a layer of flat epithelial cells, and the new tissue beneath the original dermis appeared as a thin zone of flattened, horizontally oriented fibroblasts which grew around and into the rayon threads. Capillaries were not observed in the new growth.

During the first 3 or 4 weeks after explantation (fig. 6), the epidermis and the dermis maintained this early character except for the disappearance of capillaries; the follicles remained essentially unchanged, the ridge primordia becoming slightly larger and more numerous. After this time the epidermis and dermis slowly became thinner and the follicles and ridges smaller and less numerous, though the new growth continued to increase and to show the same characteristics (fig. 7). The outer cells of the epidermis grew progressively flatter until, after 6 to 8 weeks (fig. 8), the epidermis consisted of only two or three layers of squamous cells upon a single layer of small, cuboidal basal cells. Cultures maintained for 3 months or longer usually showed a very thin epidermis without follicles or ridges and a very thin hypocellular dermis from which the denser collagen had disappeared, though the new growth continued to appear unchanged.

During all stages of growth, small pigment granules were observed in the cytoplasm of some cells in the basal layer, the rete ridges, and the follicles, and mitotic figures were observed in cells of the basal layer and the dermis.

After Inoculation With Herpes Simplex Virus

To compare the histologic appearance of inoculated cultures with that of naturally infected tissue, a number of specimens obtained by biopsy of clinical herpes simplex lesions in human patients were studied (fig. 9). These exhibited the nuclear changes, giant cells, necrosis, and vesiculation usually considered characteristic of the disease (*8–10*).

Among the cultures inoculated with herpes simplex virus and subsequently incubated at 30, 35, or 37° C, no differential effect of temperature was observed, though Wheeler noted more virus production at 35° C than at 30° C (*11*).

The histologic and cytologic changes developing in cultures after inoculation closely resembled those naturally occurring in human patients, though grossly visible herpetic vesicles were not observed. Nuclei of some epidermal cells were enlarged and pale staining; the chromatin was arranged in large granules throughout the nucleus or in a narrow zone underlying the nuclear membrane, and the nucleolus was small or absent. Where the chromatin was marginated, the remaining portion of the nucleus was occupied by a faintly basophilic, finely granular mass or by an intensely eosinophilic, smaller, granular, or homogeneous stellate mass surrounded by an unstained zone. The cytoplasm of the involved cells frequently was unstained though the cell membrane remained apparent. A number of these cells lying together gave the appearance of a fine reticulum in whose interstices were the abnormal nuclei. Frequently, the cell membranes lacked continuity in sections, so that the appearance was that of a vesicle containing nuclei and strands of the reticulum. In occasional cells the cytoplasm was swollen, homogeneous, and eosinophilic and the cell membrane was smooth, with the nucleus located centrally. These enlarged, rounded cells, sometimes several together, were free in an unstained area. Occasional cells were seen in which several closely packed, abnormal nuclei lay within an apparently normal cytoplasm.

The cellular changes appeared earliest in the intermediate zone of the epidermis, successively involved the cells of the basal layer and of the hair follicles, and sometimes the fibroblasts of the dermis as well. When the basal layer became involved, the cells lost their columnar or cuboidal shape and became irregular in outline and their parallel alignment was lost. There was frequently edema beneath the basal cells and disappearance of the basement membrane.

The earliest changes noted occurred 8 hours after inoculation, when some epidermal nuclei showed enlargement, decreased basophilia, and separation of chromatin into small strands (fig. 10). Margination of chromatin first occurred between 12 and 18 hours, and eosinophilic material occupying the central portion of the nucleus was first observed at 18 hours. These changes continued to become more widespread until by the 5th day essentially all cells of the epidermis were affected. Reticular and balloon-cell degeneration, multinucleated cells, liquefaction degeneration, and evidence of early vesicle formation were first observed on the 3d day (fig. 11), and reached their greatest prominence on the 5th day (figs. 12 and 13). After the 6th day the epidermis progressively degenerated, and most cultures were dead by the 10th day. The dermis remained essentially normal throughout except for the occurrence of nuclear changes in rare fibroblasts.

DISCUSSION AND CONCLUSIONS

In determination of the rate of growth of cultured skin, the underlying rayon grid furnished a means to readily estimate the increase in area of the tissue, but measurement of depth or of volume of tissue was not made; consequently, the actual amount of new tissue produced and the extent to which cell migration occurred were not determined. That true auxetic growth had occurred, however, was indicated by the appearance in histologic section of an increase in number of cells with no significant decrease in size, and by the presence throughout culture of mitotic figures. The attrition of this series of cultures, which averaged about 10 percent loss because of degeneration, contamination, or other cause during 1 week, and the initial survival of 85 percent of the explants, indicate that at least 25 percent of the explants cultured could be used for experimental purposes after 12 weeks.

Although no complete study of the development of fetal skin has been made, the consensus of several investigators (*6, 7, 12–16*) has been that after about 14 weeks of fetal life the primordia of the rete ridges appear, with sweat glands beginning to form at about 15 weeks, and differentiation of the stratum corneum at about 16 weeks, the hair anlagen developing after the 12th week. After culture, the skin of the explants continued to differentiate, the epidermis, the hair follicles, and the dermis having a more mature appearance after a period of culture than before, though the degree of differentiation in the cultures did not equal that in uncultured skin of an equivalent age.

The greatly thickened surface layer was apparent in all explants and may have been an accumulation of normal products which in the fetus would be scaled off into the vernix caseosa. This phenomenon has been observed by practically all investigators, and Fell and Mellanby (*17, 18*) have described the decrease and disappearance of keratin in cultures grown on medium containing excess vitamin A. The increase in thickness of the intermediate layer of the epidermis after culture may likewise be due either to a true change in the manner of growth or to a failure of the superficial cells to scale off, though the change in character of the superficial cells, the basal cells, and the basement membrane indicates that this is more than a simple retention of a normal scale.

The thinning and increased density of the dermis may be partly a result of compression of the tissue, though the increase in number and size of the cells and the formation of larger collagen fibers than could result from the earlier reticulum indicate that a true accretionary growth is taking place.

Histologic and cytologic changes developing in explants after inoculation with herpes simplex virus closely resembled those previously described in human patients, in laboratory animals, and in tissue culture (*8–10, 19–22*). The absence of dermal edema and cellular infiltration, usually seen in clinical material, is expected since no active circulation remains. Goodpasture and Anderson (*23*), in human fetal membranes grafted upon chick chorio-allantois, found a typical inflammatory reaction in the subepithelial region after inoculation with herpes virus. This is expected, since in their material the vessels of the human tissue contained chick red blood cells, which indicates an active connection between the circulatory systems of the human tissue and the chick.

Cytologic changes 8 hours after inoculation are in reasonable accord with the findings of Scott and his associates (*24*) that in tissue culture of rabbit corneal cells earliest viral changes appeared within 4 hours after inoculation. The development of intranuclear inclusions after 18 hours also agrees with the observations of Scott and others (*24, 25*), though some investigators have found these inclusions appearing only after a much longer time (*26–28*). Detailed studies of the development of herpes simplex lesions in human subjects have not been made.

The various epidermal changes seen in the explants can occur individually and in combination in other diseases, such as herpes zoster, variola, and varicella, and the so-called intranuclear inclusion bodies can be experimentally produced by a number of agents other than viruses (*29*), but the presence of all of these changes is not known to be associated characteristically with any condition other than herpes simplex. The results of the present study thus reinforce the implication of Bang and Niven (*4*) that this system offers a better means for analysis of disease processes, as contrasted to infection, and for the study of details of pathogenesis, virus specificity, and host-parasite relationship in the absence of the influence of circulation.

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PLATE 9

FIGURE 1.—Culture #493, incubated for 10 days. Explant prepared from fetus #31-D, a 13-week fetus shipped from Copenhagen and cultured 58 hours after death. × 30

FIGURE 2.—Culture #493, incubated for 62 days. × 30

FIGURE 3.—Skin from fetus #31-D, 58 hours after death. Hematoxylin and eosin. × 100

FIGURE 4.—Skin from fetus #41-D, an 18-week fetus shipped from Copenhagen, 60 hours after death. Hematoxylin and eosin. × 100

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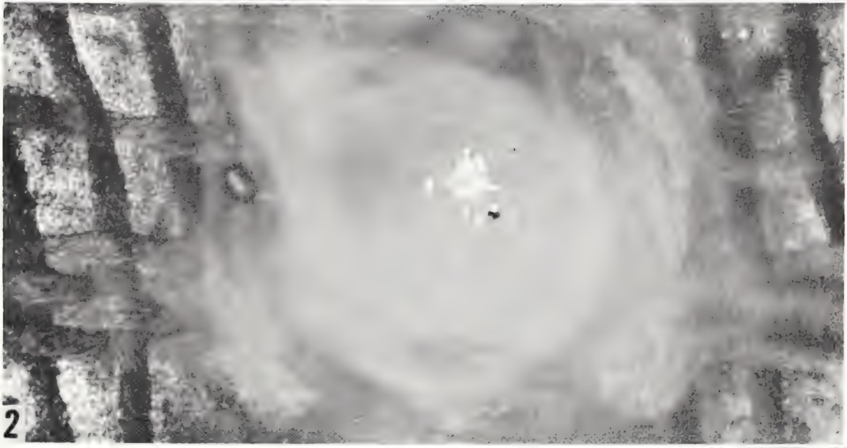

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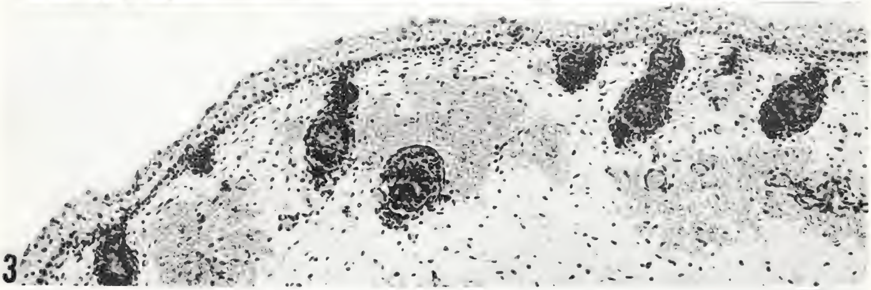

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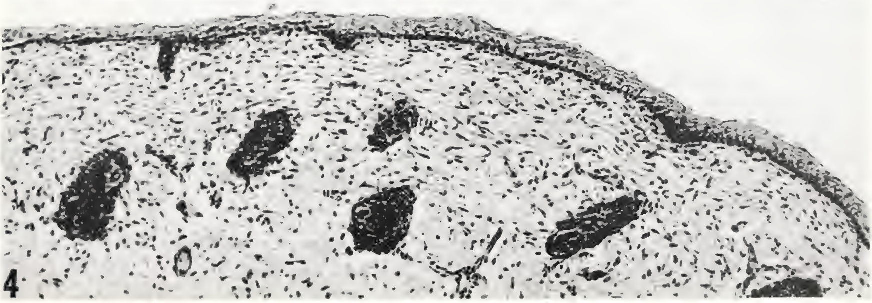

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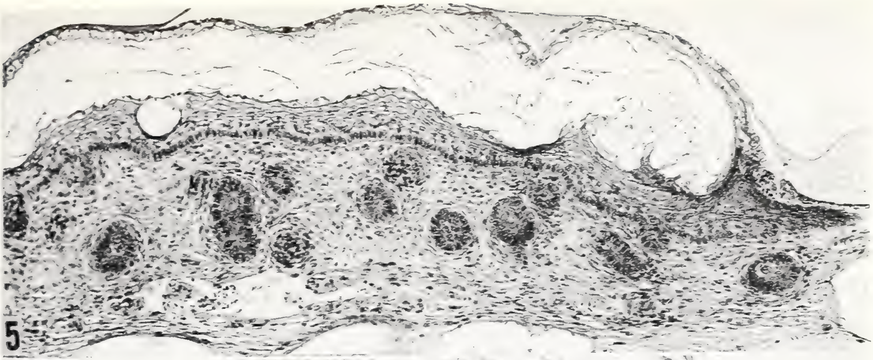
Plate 10

Figure 5.—Culture #494 from fetus #31-D, incubated for 10 days. Hematoxylin and eosin. × 100

Figure 6.—Culture #500 from fetus #31-D, incubated for 20 days. Hematoxylin and eosin. × 100

Figure 7.—Culture #492 from fetus #31-D, incubated for 30 days. Hematoxylin and eosin. × 100

Figure 8.—Culture #493 from fetus #31-D, incubated for 62 days. Hematoxylin and eosin. × 100



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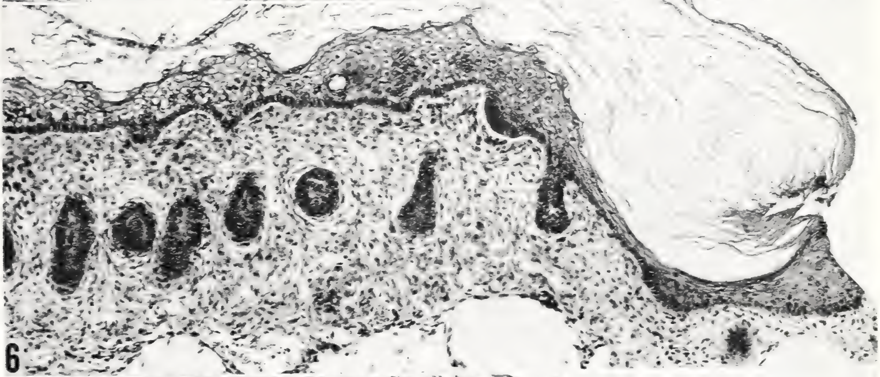

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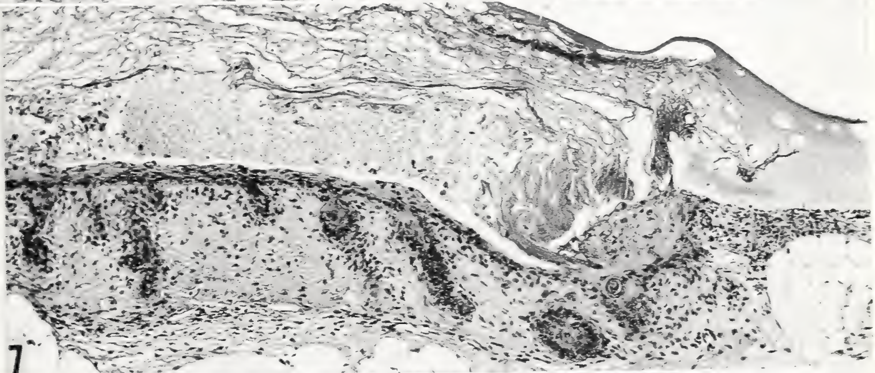

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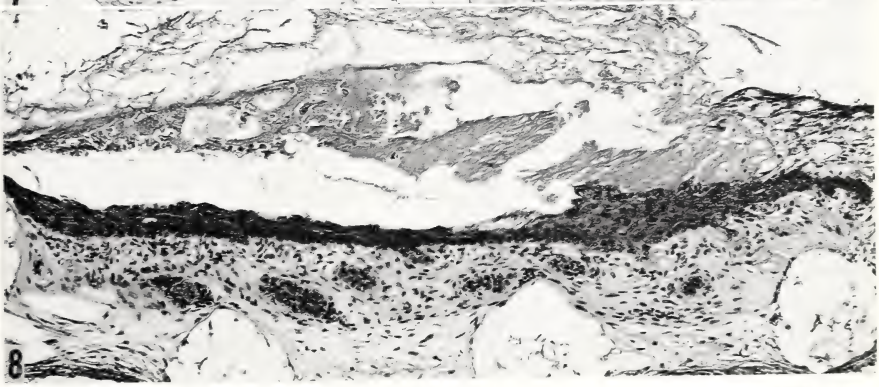

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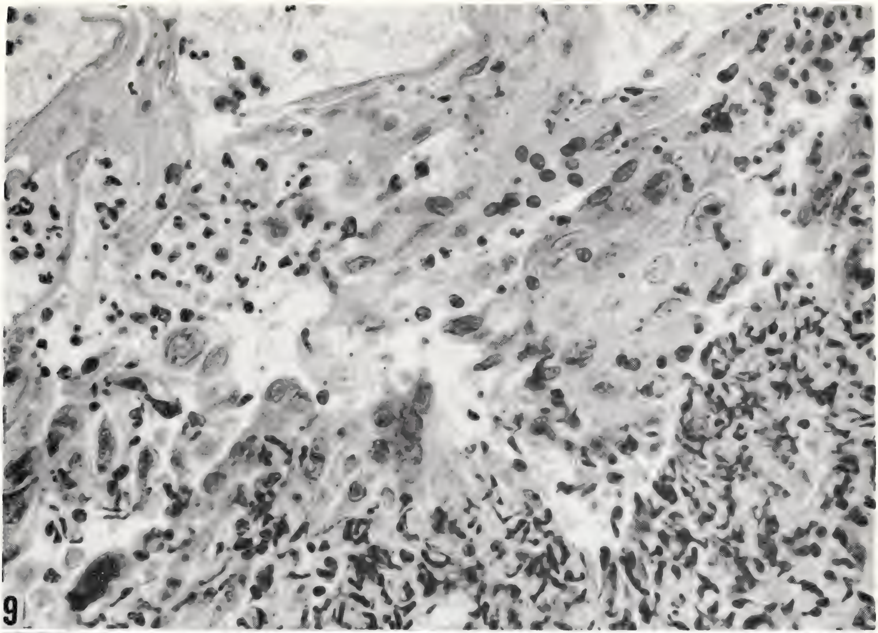
PLATE 11

FIGURE 9.—Biopsy of herpes simplex lesion from adult male, 3 days after appearance of lesion. *Note* necrosis and vesiculation, intranuclear lesions in epidermal cells, cellular infiltrate in dermis appearing in *lower portion* of photograph. Hematoxylin and eosin. × 400

FIGURE 10.—Culture #792 from fetus #48-D, a 14-week fetus shipped from Copenhagen. Eight hours after inoculation with herpes simplex virus on 5th day of culture. *Note* enlargement of epidermal nuclei and early granulation of chromatin. Hematoxylin and eosin. × 400

FIGURE 11.—Culture #643 from fetus #35-D, a 13-week fetus shipped from Copenhagen. Three days after inoculation with herpes simplex virus on 6th day of culture. *Note* reticular degeneration and early vesiculation in central epidermis, liquefaction degeneration of basal-cell layer, and numerous intranuclear lesions. Hematoxylin and eosin. × 400



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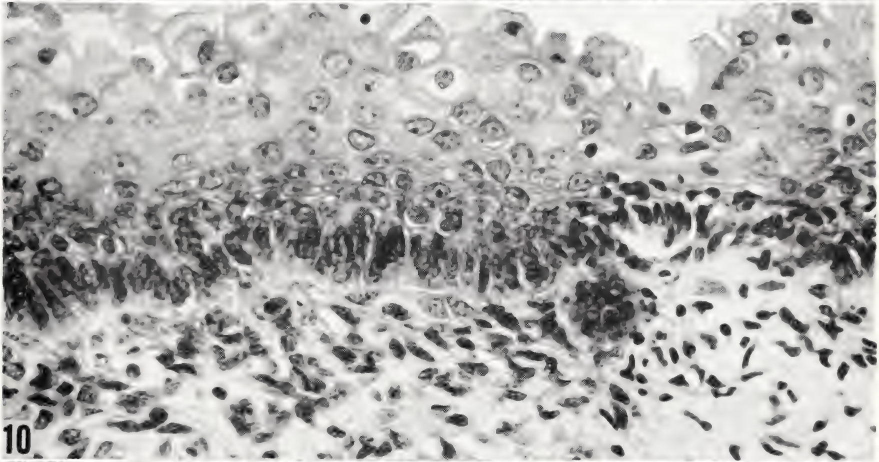

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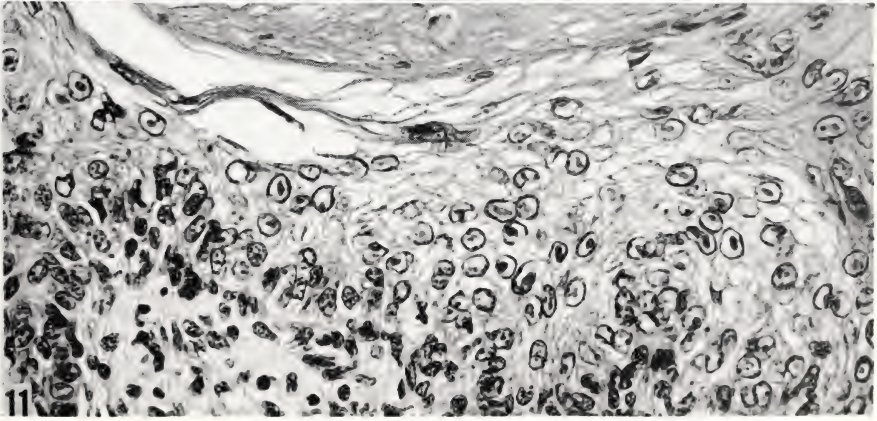

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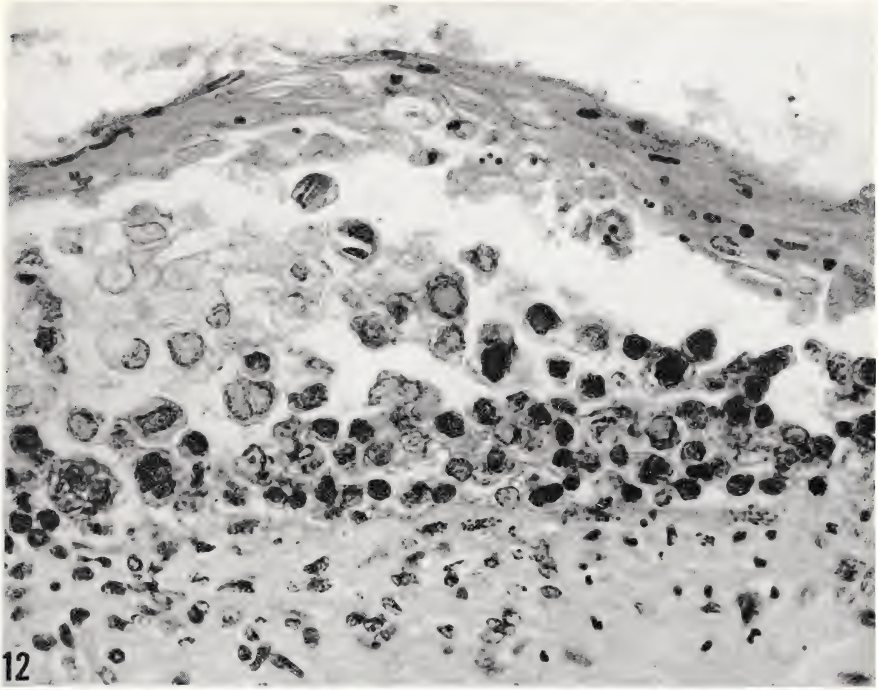
Plate 12

Figure 12.—Culture #688 from fetus #40-D, a 13-week fetus shipped from Copenhagen. Five days after inoculation with herpes simplex virus on 25th day of culture. *Note* necrotic changes and vesiculation, multinucleate cells, intranuclear lesions. Hematoxylin and eosin. × 400

Figure 13.—Lower magnification of figure 12 that shows vesiculation in two sites. Hematoxylin and eosin. × 100



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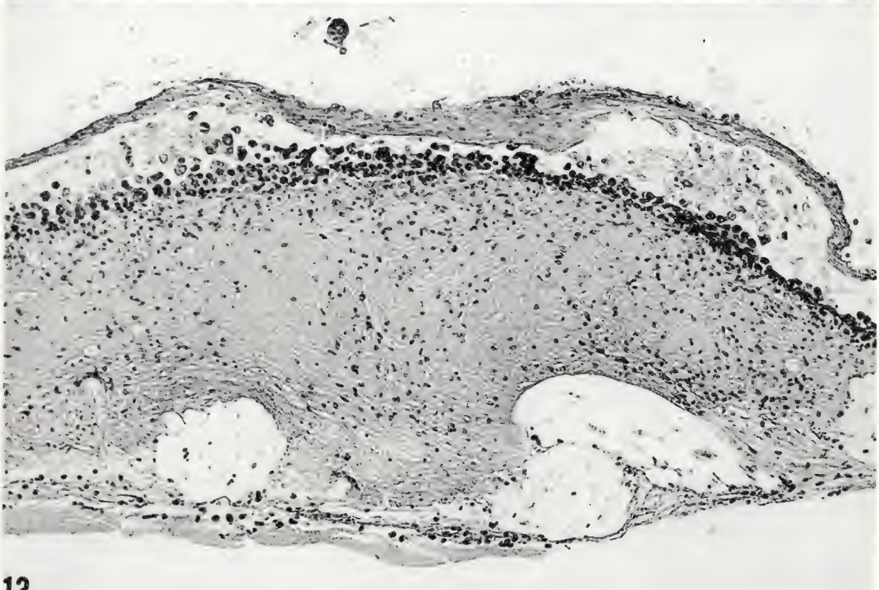

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DISCUSSION

RONALD W. GILLETTE, Ph.D., Department of Plastic Surgery, The New York Hospital, Cornell Medical Center, New York, New York

Dr. McGowan is to be congratulated on his comprehensive and detailed study of the long-term organ culture of fetal skin and its applications to the study of virus infection. In his presentation, Dr. McGowan pointed out that organ-culture techniques offer the salient advantage of permitting studies on pathogenesis in tissues completely isolated from humoral control. I would like to stress that this idea could be expanded to include studies on normal tissue functions and on the reactions of normal tissues to hormones and other physiologically active agents.

Dr. McGowan used fetal skin in his research and I think it might be worth while to compare his results with some that we obtained from the organ culture of adult skin. I was particularly interested to note that Dr. McGowan could maintain viable fetal skin, by his culture method, for 3 to 6 months. Our organ-culture method differs somewhat from his and so do our results. In brief, our technique involves floating adult mouse skin, dermal surface downward, on a complete, liquid tissue-culture medium (*1*). We use skin from the outer surface of the mouse's ear routinely, because it provides extremely thin full-thickness skin amenable to floating and diffusion of nutrients. However, adult human skin has been successfully cultured by this method in a few instances. Cytological studies of a large number of organ cultures revealed that untreated adult skin retains its structural integrity in this organ system for about 2 weeks at most. After this time, histologic sections of the skin showed that its architecture had altered radically and concurrent observations of the cultures revealed that the floors of the culture flasks were covered with healthy, proliferating, modulated cells that had dropped from the floating organ culture. These sheets of cells bore little resemblance to organized skin. Even the addition of anti-inflammatory agents, such as cortisone acetate, enables us to maintain organized adult skin for only about 4 to 5 weeks (*2*). The adult skin passes through the same histological stages of dedifferentiation that Dr. McGowan describes for fetal skin, but these changes occur at a much accelerated rate. These results re-emphasize the basic differences between cultures of adult and fetal skin and the dangers inherent in the application of conclusions drawn from one to the other.

Another point I should like to consider is that there is still a great need in the field of organ culture for a test system which will enable us to distinguish between "structural integrity" and "functional viability." Organ cultures may retain their cellular integrity and general architecture, as judged by histologic examination, while their original organ function *in vivo* may have been severely impaired. We test the functional viability of adult mouse auricular skin, after maintenance in organ culture, by grafting it to the dorsum of the original donor animal. As I have mentioned, we found that *in vitro* treatment with appropriate steroids increases the length of time that adult skin can be maintained in organ culture; it also increases the percentage of organ cultures that can be grafted successfully. However, we discovered that the histologic appearance of the skin could be an extremely misleading criterion for estimating functional ability. Figure 1 shows a section of mouse auricular skin that had been treated in organ culture for 3 weeks with cortisone acetate. Based on my experience with a large number of these cultures, I can safely state that such skin, despite the obviously severe architectural changes, would function as a successful autograft in approximately 90 percent of the cases. Figure 2 shows a section of skin treated in organ culture with a combination of cortisone acetate and deoxycorticosterone acetate for 3 weeks. Again, based upon my experience, I can predict that, in spite of its rather good histologic appearance, this skin would not have functioned as a successful autograft. Skin treated with this combination of steroid agents retained its structural integrity, but lost the capacity to resume its original function *in vivo*.

Dr. McGowan's work with the herpes simplex virus might lead to another test of functional viability, if some viruses are sufficiently selective to be dependent only on unmodulated adult cells. Another area from which a test could be developed, and which has been explored to some extent by several investigators, is endocrine function. The capacity of endocrine tissue to resume its function when replaced in animals deprived of endocrine glands could be utilized as a test of functional ability. Preliminary histochemical studies on organ cultures from our laboratory suggest that the histochemistry of enzyme systems may yield valuable information on the degree of modulation of adult skin *in vitro*.

Certainly any test method giving investigators a definite indication of the point at which cultured tissue loses so much of its functional ability that organ cultures have been transformed into tissue cultures would be enormously valuable. In fact, without these parameters, most of us who are engaged in organ culture are working half blindly.

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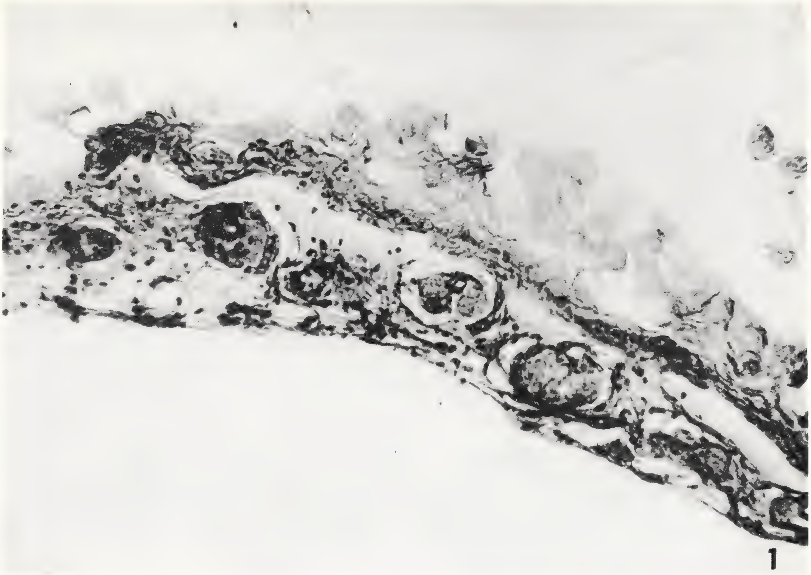
PLATE 13

FIGURE 1.—Auricular skin from a strain A mouse after maintenance for 3 weeks in organ culture in complex medium and cortisone acetate. Our experience indicates that such skin would function as a successful autograft. Hematoxylin and eosin. × 160

FIGURE 2.—Auricular skin from a strain A mouse after maintenance for 3 weeks in organ culture in a complex medium with cortisone acetate and deoxycorticosterone acetate. *Note* that, despite good appearance of skin, our experience indicates that it would not function as an autograft. Hematoxylin and eosin. × 160



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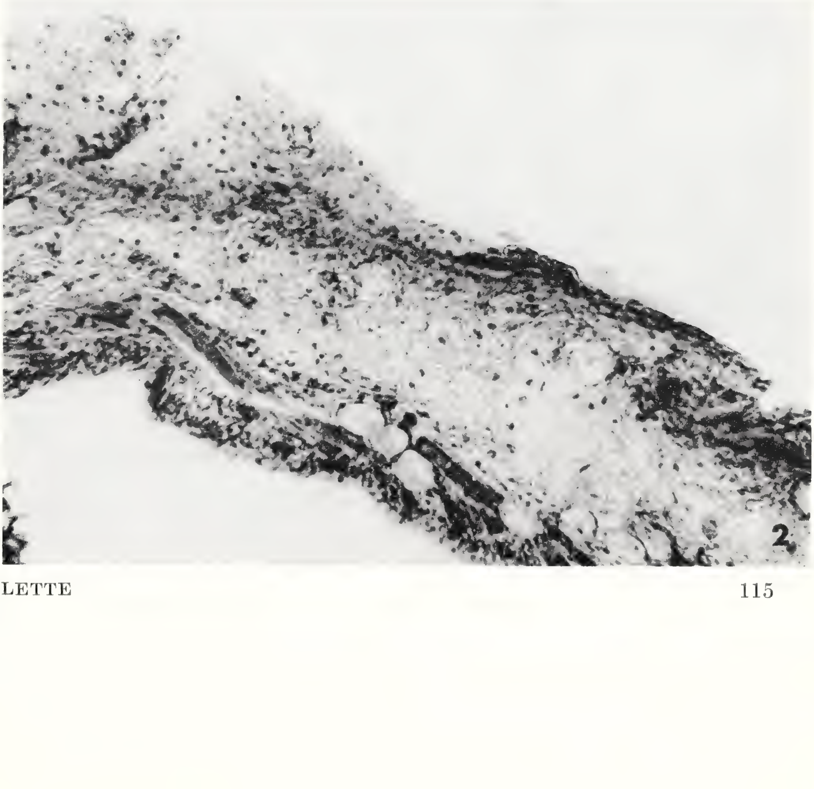

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ANTIBODY PRODUCTION IN ORGAN CULTURES OF LYMPH-NODE FRAGMENTS FOLLOWING *IN VITRO* SECONDARY ANTIGENIC STIMULATION[1, 2]

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SUMMARY

The technique for obtaining a secondary antibody response *in vitro* by stimulating cultured fragments of immune rabbit lymph nodes is described. A thin pad of glass wool overlying the fragments in a Leighton tube was used to hold the fragments in place and to replace plasma for this function. In addition, the glass wool fibers provided an increased surface area for cellular migration, which may be studied in living cultures or in fixed and stained preparations. Experiments leading to the development of chemically defined medium are described; the components of this medium that appear to substitute for serum include hydrocortisone and insulin. Studies on the inhibition of antibody production *in vitro* by bacteriostatic levels of chloramphenicol suggest that the drug acts at an early step which is probably peculiar to the initiation of the secondary response.—NCI Monogr. 11: 117–126, 1963.

SEVERAL YEARS ago Dr. Maria Michaelides and Dr. Albert H. Coons devised a method of stimulating the secondary response in organ cultures of immune rabbit lymph-node fragments (*1*). The work to be described here deals with two quite different applications of this culture technique. One has been to determine optimal nutritional requirements of this culture system first by finding a defined medium which enables some antibody production to occur *in vitro* and next by supplementing with known compounds this basic medium to enhance production further. The second application has been to study the effect of chloramphenicol on the inhibition of this *in vitro* response. These studies will soon be published in detail elsewhere.

MATERIALS AND METHODS

The basic technique originally devised is as follows: Normal adult male rabbits receive an injection in each hind foot pad of 10 to 12.5

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[1] Presented at the 13th Annual Meeting of the Tissue Culture Association, Washington, D.C., May 29–31, 1962.

[2] Supported by research grant H-2255, National Heart Institute, National Institutes of Health, Public Health Service.

[3] The assistance of Miss Linda Chesnel and Mrs. Elaine Lacks is gratefully acknowledged.

mg bovine serum albumin (BSA) and 100 to 167 Lf diphtheria toxoid—both antigens being dissolved in the same 0.1 ml of saline. Three to 12 months later, when the rabbit's circulating titers to these two antigens are low, the lymph nodes draining the injection sites are removed aseptically and cut into 1 mm^3 fragments. These fragments are incubated for 2 hours with a dilute antigen solution of 0.5 mg BSA and 5 Lf diphtheria toxoid per ml of medium and are then rinsed several times with Hanks' solution to remove excess antigens. This step constitutes the *in vitro* secondary stimulation. Generally a small group of fragments is simultaneously incubated in medium without antigens and serves as the nonstimulated controls. Twelve fragments are implanted in rabbit plasma (usually autologous plasma) in a roller tube. Each tube receives 1 ml of basal medium[4] containing 25 percent normal rabbit serum. The tubes are incubated at 37° C in a roller drum. The medium is replaced every 3 days and saved for later measurement of its antibody titer. Stimulated lymph-node cultures of this sort frequently continue to produce high titers of antibodies simultaneously to BSA and diphtheria toxoid for over 3 weeks. Finally, the antibody titers are measured by the hemagglutination method with tanned sheep erythrocytes [Stavitsky's modification of the Boyden procedure (*2*)].

The only significant deviation from this original technique has been replacement of the plasma clot by glass wool pads to hold the fragments in place. A thin pad of glass wool, precut to the shape of the inner flat surface of a Leighton tube, is inserted over the fragments once they are aligned in the tube. This inert matrix of spun glass contributes no nutritional factors to the culture, but fixes the fragments in place and provides increased surface area onto which cells may migrate out from the fragments. The live culture may be examined under low- and medium-power objectives any time during an experiment. Also the fragments and their outgrowth entangled in the glass wool may be fixed and stained like coverslip preparations. Plasma may be added to the glass wool, which also prevents loss of fragments from lysis and retraction of the clot.

DEVELOPMENT OF A CHEMICALLY DEFINED CULTURE MEDIUM

In the culture system, as originally devised, there are three uncontrolled variables affecting the titer of antibody produced. The first and most significant, of course, is the starting tissue itself, which must be obtained fresh from a rabbit for each experiment. The lymph-node fragments differ considerably from animal to animal in two respects: 1) in the degree of their primary *in vivo* antigenic stimulation and 2) in their potential for protein synthesis as a functon of intrinsic nutritional factors. The existence of this second difference seems apparent from the fact that fragments from some rabbits produce moderate amounts of antibodies when cultured in mere basal medium, *i.e.*, medium free of serum, while fragments from other rabbits in the identical basal medium often show no response. Yet both sets of fragments produce good antibody responses in serum-containing medium, indicating they were both equally well primed by the initial *in vivo* antigen stimulation. A possible explanation for different responses in basal medium is the existence of an intrafragment pool (probably intracellular pool) of some nutritional factor(s) in the nodes of one rabbit and not in those of another.

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[4] Basal medium consists of 1 percent Eagle's amino acid mixture, 1 percent Eagle's vitamin mixture, 2.0 mM glutamine, Hanks' salt solution, glucose (75 mg/100 ml), phenol red (0.002 g/100 ml), 50 units per ml penicillin, and 50 μg per ml streptomycin sulfate. The final *p*H is adjusted to 7.4 with a sodium bicarbonate solution.

The remaining two variables affecting the *in vitro* response are the plasma clot and the serum used in the medium—both of which differ in the nutritional substances they contribute to the culture. We can eliminate the problem of plasma by replacing it with the glass wool pads described. Our interest in developing a completely defined mixture of substances to replace serum arose in the following indirect manner:

Studies preliminary to experiments on the incorporation of isotopically labeled amino acids in stimulated lymph-node cultures revealed that serum dialyzed ostensibly to remove free amino acids could not support antibody production. This deficiency could not be relieved by the presence of Eagle's mixture of 13 essential amino acids or by the addition of any of the "nonessential" amino acids. However, a serum dialysate could reconstitute the dialyzed serum to a considerable degree. In fact, the dialysate allowed good antibody production to occur in the absence of any dialyzed serum with its various proteins. More surprising, however, was an experiment in which basal medium with 12 percent normal rabbit serum allowed excellent antibody production, with 25 percent dialyzed rabbit serum allowed essentially no antibody production, and with the combination of 25 percent dialyzed serum plus 12 percent normal serum allowed practically no antibody production. One possible explanation consistent with these results is that serum contains a nutritionally essential component (or components) which is dialyzable and exists both in a free form and partially bound to a nondialyzable serum protein. On exhaustive dialysis[5] both the free and protein-bound fractions are lost from the serum. The dialyzable components in the dialysate can be returned to the dialyzed serum to render it again able to support antibody production, or the dialyzed component by itself can sustain antibody production independent of serum proteins. Furthermore, dialyzed serum may conceivably "deplete" normal serum if the dialyzed proteins of the former tightly bind the free components of the latter.

Certain hormones have these necessary properties, *i.e.*, they are dialyzable and exist in serum both free and bound to proteins. Three common ones are insulin (*3*), triiodothyronine (*4*), and hydrocortisone (*5*). We prepared a special mixture consisting of these three readily available hormones. At the time of these experiments we were also interested in the effects of vitamin B_{12}, several fat-soluble vitamins, and the nonessential amino acids. When this empirical mixture of 16 components [6] was added to the basal medium, this now serum-free medium resulted in production of antibody titers as high as did the basal medium supplemented with the serum dialysate or even with 25 percent normal rabbit serum. The next several experiments indicated that of the 16 substances included in the empirical mixture the hydrocortisone sodium succinate and the insulin (to a lesser degree) were the major active components (text-fig. 1). While individually neither the nonessential amino acids nor the special vitamin mixture sustained antibody production, yet some degree of synergism between them and the hydrocortisone and insulin was evident in still later experiments. The nature of the synergism, the range of optimal concentration of hydrocortisone and insulin, and the recognition of other dialyzable factors in serum are currently being investigated.

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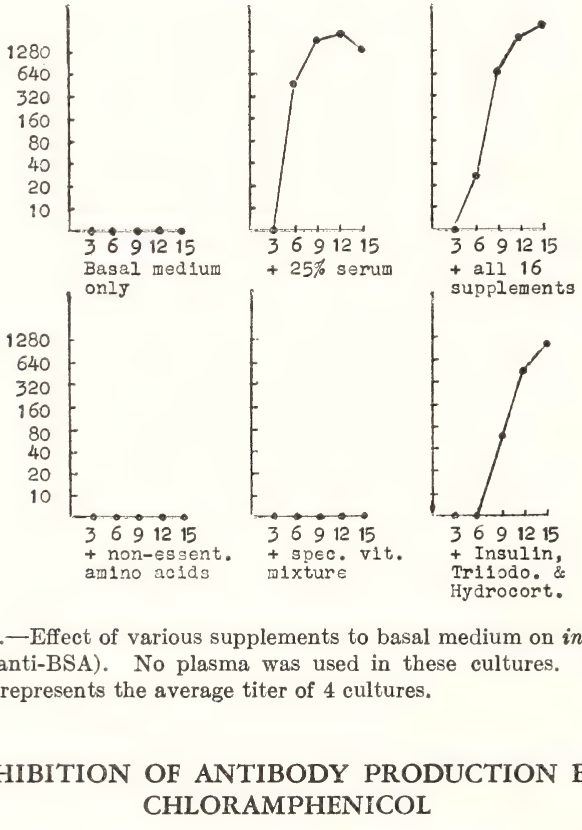
[5] In these experiments serum was dialyzed against running tap water for 72 hours at 5° C and then against several changes of normal saline-phosphate buffer at *p*H 7.0 over 24 hours at 5° C. We obtained the dialysates by dialyzing serum against an equal volume of distilled water with gentle shaking for 24 hours at 5° C.



TEXT-FIGURE 1.—Effect of various supplements to basal medium on *in vitro* antibody production (anti-BSA). No plasma was used in these cultures. Each point in these graphs represents the average titer of 4 cultures.

INHIBITION OF ANTIBODY PRODUCTION BY CHLORAMPHENICOL

Our studies with chloramphenicol require a brief summary of some relevant data from the literature. In susceptible bacterial cultures and in cell-free bacterial systems the addition of 2 to 20 μg per ml of chloramphenicol results in the prompt and nearly complete suppression of protein production (*6*, *7*). On the other hand, in mammalian cells this level of the drug has had no demonstrable effect. Indeed, only with very high levels (300 to 2500 μg/ml) has there been shown to be even partial inhibition of protein synthesis in mammalian cell cultures (*8*, *9*), in isolated thymus nuclei (*10–12*), or in cell-free systems (*9*, *13*). This apparent resistance of mammalian cells and systems to "bacteriostatic" levels of chloramphenicol has puzzled investigators interested in protein synthesis. However, we have found a clear effect of such low levels of chloramphenicol on the secondary response (*14*).

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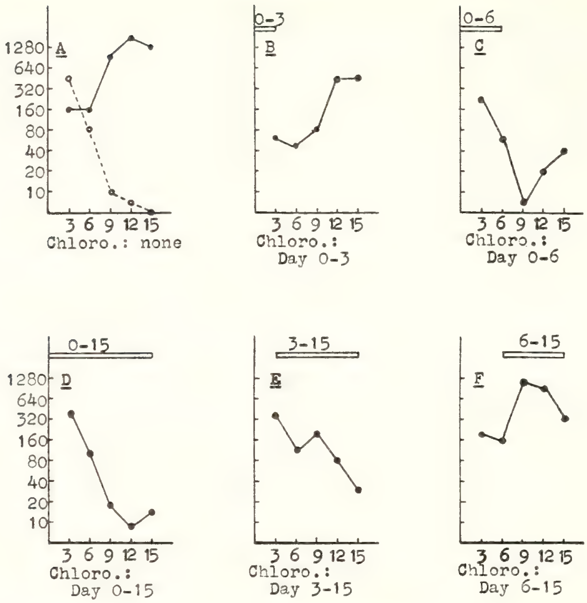
[6] The 16 components of the empirical mixture included: 0.1 mM of each of the 6 nonessential amino acids (alanine, aspartic acid, asparagine, glycine, proline, and serine), 0.25 μg per ml vitamin A, 10 μg per ml vitamin E, 0.45 μg per ml vitamin K, 5 μg per ml linoleic acid, 5 μg per ml linolenic acid, 5×10^{-6} μg per ml thioctic acid, 2×10^{-4} μg per ml vitamin B_{12}, 0.01 μg per ml triiodothyronine, 0.1 μg per ml hydrocortisone sodium succinate, and 1.0 unit per ml crystalline zinc insulin.

In one experiment various concentrations of chloramphenicol (5, 10, 20, 35, and 50 μg/ml) were included in the medium for the 2 weeks the cultures were followed. In cultures maintained in medium free of chloramphenicol, the lymph-node fragments produced titers averaging at their peak 1/5120 for anti-BSA and 1/320 for antidiphtheria toxoid. Increasing concentrations of chloramphenicol in the medium produced correspondingly more complete inhibition of antibody response. The anti-BSA response was inhibited over 95 percent by 20 μg per ml and over 99 percent inhibited by 50 μg per ml. The anti-diphtheria toxoid response, which in general was several twofold dilutions lower than the corresponding and simultaneous anti-BSA response, was completely suppressed by concentrations greater than 20 μg per ml.

Subsequent experiments showed that once the secondary response was well established, antibody production could not be appreciably stopped by a level of chloramphenicol which was completely inhibitory when continuously present in the medium from the time of the secondary stimulation. In text-figure 2 are plotted data of a typical experiment in which 50 μg per ml chloramphenicol was added to the medium on day 0 for various intervals or was added at different times after the secondary stimulation and maintained in the medium until the end of the experiment. The response of the cultures not receiving secondary stimulation *in vitro* and not exposed to the drug is shown as a *dotted* line in text-figure 2A. In these unstimulated cultures there was an initial high titer that rapidly fell in successive changes of medium; this initial peak represented the waning primary response to the antigen injected into the foot pad many months before. The response of the cultures stimulated *in vitro* and not exposed to the drug is depicted in this same figure as the *solid* line; a typical secondary response resulted in a peak antibody production around day 12. Stimulated cultures exposed to chloramphenicol throughout the 15 days (text-fig. 2D) showed a precipitous fall in antibody titers, which paralleled that in the unstimulated cultures. Exposure for the first 3 days only (text-fig. 2B) resulted in a delayed response which never quite reached that in the unexposed group of cultures. Exposure for the first 6 days (text-fig. 2C) caused a steep fall in titers, but a small response did occur 3 days after withdrawal of the drug from the medium. Exposure beginning on day 3 and continuing to the end of the experiment (text-fig. 2E) resulted in a slower fall in titer than in the cultures continuously



Text-figure 2.—Effect of 50 μg per ml chloramphenicol on *in vitro* antibody production (anti-BSA). Chloramphenicol was present in the culture medium for various intervals starting at different times as indicated by the *bar* at the *top* of each graph. Each point represents the average titer of 3 to 4 cultures.

exposed for all 15 days. Finally, exposure beginning on day 6 (text-fig. 2F) allowed antibody production to continue for the next 6 days as well as it did in the unexposed control group of cultures.

The general pattern of response to chloramphenicol described in this experiment has been repeated in numerous others of the same design. These studies indicate that levels of chloramphenicol, which suppress protein synthesis in bacteria, also markedly inhibit antibody production in mammalian cells in culture when the exposure to the drug includes the period of 9 or 12 days immediately after the *in vitro* antigenic stimulation. These data suggest that chloramphenicol acts at a point in the metabolic sequence for antibody synthesis peculiar to the initiation of the secondary response.

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DISCUSSION

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Dr. Ambrose has reported some interesting and unique findings. Discussion of them is complicated by the following considerations: 1) There is some question about what he is measuring with his antibody assay and 2) he is studying phenomena whose mechanisms are poorly understood. Let me be more explicit.

The hemagglutination method measures antibody activity and is probably also a rough measure of antibody concentration. It is, therefore, possible that some of the antibody in the medium was not recently synthesized, but was made at some time in the past and released into the medium after the disruption of antibody-containing cells. The distinction between *de novo* synthesis of antibody and release of antibody can be made with certainty only by measurement of the extent of incorporation of radioactive amino acids into antibody. Table 1 illustrates the results of an experiment in which the secondary antibody response of the rabbit lymph node was stimulated *in vivo* and the node was set up in *in vitro* culture as in the experiment of Dr. Ambrose; the only exception was that the fragments of node were pinned to a stainless-steel wire grid with agar. It is clear that in this situation, which is admittedly quite different from Dr. Ambrose's, the hemagglutination titer is not a reliable measure of *de novo* antibody synthesis. I do not know why it is not a reliable gauge of synthesis, but suppose that in the presence of these purine and pyrimidine analogues some disruption of cells or cell permeability occurs, with release of intracellular antibody which was not made recently. However, over a period of years, we have tried with only intermittent success to get incorporation of C^{14} glycine into the antibody which appears in the medium in a system like the one Dr. Ambrose used. We have succeeded on occasion and I must, therefore, conclude that *de novo* antibody synthesis can occur under these conditions. That incorporation does not occur more consistently

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[1] This investigation was supported by research grant E-1865 from the Allergy and Infectious Diseases Institute, National Institutes of Health, Public Health Service.

[2] The assistance of Mr. Weldon W. Harold is acknowledged.

must be a reflection of the relatively small amounts of antibody which are synthesized. Indeed it would be difficult to explain the data on any other basis; it is unlikely that the antibody which appears in the medium a few days to a week after the addition of antigen represents antibody that has persisted intracellularly from the last injection of antigen 3 to 12 months before. The rub, as I see it, comes in the interpretation of the titers of cultures 2 to 3 weeks old. The possibility remains that the antibody in the medium at this time represents a combination of newly produced antibody and antibody synthesized previously and released into the medium. A more sensitive isotopic assay and the development of conditions favoring the production of larger quantities of antibody are urgently needed.

TABLE 1.—Hemagglutination versus incorporation assay of *in vitro* antibody synthesis in presence of inhibitors

Lymph node* in medium plus	Hemagglutination titer†	cpm‡
—	640	1, 488
500 μg 5-BRUD§	320	198
200 μg 6-AUR§	320	242
200 μg 8-AA§	320	284
200 μg 6-AT§	160	290

*Popliteal lymph nodes removed 3 days after third injection of bovine γ-globulin into hind foot pads of rabbit. Cultures set up of lymph-node fragments on stainless-steel grids in 1 ml of medium for 48 hours.

† Reciprocal of hemagglutination titer.

‡ Counts per minute of specific bovine γ-globulin-anti-γ-globulin precipitates.

§ 5-BRUD = 5-bromodeoxyruidine; 6-AUR = 6-azauracil riboside; 8-AA = 8-azaadenine; 6-AT = 6-azathymine.

Dr. Ambrose is studying the *in vitro* induction of the secondary antibody response, followed by continued intracellular synthesis of antibody and secretion of this antibody into the medium for a period of weeks. He has made some very useful observations on the factors in the medium that favor the secondary antibody response. However, it is difficult to know at what point(s) in this sequence of reactions the factors in serum, the special mixture and hormones, operate. In short-term, 24- to 48-hour cultures of lymph nodes cultured after *in vivo* induction of antibody synthesis, we found that optimal concentrations of certain amino acids had to be present in the medium for maximal synthesis (*1*). Addition to the medium of serum, serum ultrafiltrate, chick-embryo extract, purines and pyrimidines, certain lipides, carbohydrates, and coenzymes did not promote antibody synthesis in these cultures (*1*). In a similar study with spleen, Mountain (*2*) found that purines and pyrimidines, chick-embryo and liver extract, certain vitamins, and sucrose did not enhance antibody formation. Higher concentrations of sucrose, cysteine, cystine, and reduced glutathione inhibited antibody production (*2*). Working with isolated spleen cells, Vaughan *et al.* (*3*) added ribonucleosides, glucose, and ascorbic acid to the medium without affecting antibody synthesis, but higher concentrations of ascorbic acid, glutamine, and nucleosides inhibited antibody production. The nutritional requirements for successful long-term cultures are less well defined. From the work of Trowell (*4, 5*) on organ cultures of rat lymph nodes *in vitro,* it appears that a simple synthetic medium consisting of certain amino acids, inorganic salts, glucose, thiamine, paraaminobenzoic acid, and insulin supports lymphoid cells in good condition as well as differentiation of reticulum cells to large lymphocytes. Dr. Ambrose also obtained antibody synthesis in the absence of serum if the special mixture was added to the basal medium.

Dr. Ambrose's findings on the effects of chloramphenicol on antibody formation in a mammalian cell system are unique. It is interesting that Trowell (*4, 5*) had 0.06 percent chloramphenicol in his cultures—an amount greater than the highest concentration used by Dr. Ambrose—without any apparent effect on morphology or differentiation in his system. It would, therefore, be of interest to find out what biochemical, morphological, and physiological effects chloramphenicol has on the *in*

TABLE 2.—Synthesis of antibody in long-term cultures of rabbit lymph nodes

Days in culture*	cpm†
2	890
3	1, 070
5	760
7	820

*Rabbit popliteal lymph-node fragments derived from rabbits 3 days after the second injection of diphtheria toxoid. One μc of C^{14}-glycine was placed in the medium. Medium and isotope were replaced on days 2, 3, 5, and 7 of culture.

†Counts per minute of specific antigen-antibody precipitate derived from tissue-culture medium.

vitro antibody-forming system. I would agree that the data certainly suggest an effect on the inductive phase of the *in vitro* antibody response.

Therefore, further studies of normal and antibody-forming lymph-node cultures are a glaring requirement. I am perplexed about what goes on in the systems we have studied in which lymph nodes are removed from the rabbit 3 days after the second injection of antigen and then cultured in a manner similar to Dr. Ambrose's system, with the exception that the tissue fragments are stuck to a stainless-steel wire grid with agar. Under these conditions antibody synthesis continues for at least 1 to 3 weeks at a slowly declining rate, as shown in table 2. There is little evidence of mitosis *in vitro*, which is reminiscent of Trowell's observations on cultures of normal rat lymph nodes (*4*, *5*). Nevertheless, as shown in table 1, purine and pyrimidine analogues inhibit antibody synthesis almost completely and studies of *in vitro* incorporation of P^{32} into various cell fractions suggest a greatly increased synthesis of phospholipide, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) [table 3 (*6*, *7*)]. It is therefore surmised that these antibody-forming tissues are synthesizing and turning over substances other than antibody. Moreover, there seem to be important differences between antibody synthesis as it occurs *in vivo* and *in vitro*. The peak of antibody synthesis *in vivo* is 3 to 4 days after the second injection of antigen and by the 6th day only a low level of synthesis persists (*8*). Yet, lymph nodes removed during the peak of maximal synthesis continue to make antibody at only a slowly declining rate for 1 to 3 weeks *in vitro*. The difference may be that *in vivo* the immature plasma cell, which is capable of maximal antibody synthesis, differentiates into the mature plasma cell that is capable of only minimal synthesis, whereas *in vitro* this differentiation may not occur.

That Dr. Ambrose's studies raise more questions than they answer is a compliment rather than a criticism. I am certain that more detailed cytochemical, biochemical, and immunological studies of what is going on in his cultures will provide much useful information as well as many more incisive questions. Thus is progress made. The difficulty of the use of hemagglutination titration to measure *de novo* antibody synthesis in *in vitro* lymph-node cultures is stressed. It is, however, concluded that such synthesis does occur *in vitro* after the addition of antigen to lymph-node fragments from rabbits which were stimulated for the first time many months previously.

TABLE 3.—Incorporation of inorganic P^{32} into phospholipides, deoxyribonucleic acid, and ribonucleic acid of lymph nodes

Source of node*	Phospholipides (% dose/mg P)	RNA	DNA
Normal rabbit	3. 7	3. 0	0. 43
Normal rabbit	2. 3	1. 2	0. 26
Immune rabbit	6. 4	4. 6	1. 05
Immune rabbit	5. 6	4. 4	0. 71

*Immune rabbit—node removed from rabbit 3 days after second injection of diphtheria toxoid. Lymph-node slices incubated with inorganic 50 μc P^{32} for 5 hours *in vitro*.

Further biochemical, cytochemical, and immunochemical studies are required to understand what is occurring in organ cultures of antibody-forming tissues.

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THE CORTICOTROPHIC ACTIVITY OF THE ANTERIOR HYPOPHYSIS *IN VITRO* [1]

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SUMMARY

Explants of the anterior hypophysis of young rats were cultured on a coagulated medium of human plasma and human fetal brain extract diluted with Hanks' salt solution. Studies were made of the morphology of these organ cultures and of their production of corticotrophic hormones. The functional activity was measured by estimation of the corticosteroids in the medium when these cultures were combined with explants of adrenal cortex. This was correlated with the morphological changes elicited in the cortical cells of the adrenal culture by the adjacent hypophyseal culture. The morphological and functional effects on the cortical cells occurred during the first 2 weeks, thereafter diminishing. Degranulation of the hypophyseal cells preceded the decrease of the functional activity. The addition of fragments of the medial eminence to these combined organ cultures did not prolong the production of corticotrophin. Implantation in the sella turcica of hypophysectomized rats of a culture that had lost its granules and its functional activity resulted in growth with a return of function and a reappearance of intracellular granules; this may mean that the hypothalamus has a humoral trophic effect on the cells of the anterior hypophysis.—NCI Monogr. No. 11: 127–141, 1963.

THE ANTERIOR hypophysis lends itself well to organ culture (*1–3*). This technique, as distinct from ordinary tissue culture, allows the study of functional activity. Since there is also a maintenance of architectural characteristics, we can attempt the correlation of form and function. Finally, it is possible by growing explants close to each other to observe the mutual influence of different organs, and within the endocrine system to study the effect of a hormone-producing tissue on a target tissue, for example, the corticotrophic activity of the anterior hypophysis as reported here.

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[2] The author expresses his sincere appreciation to Miss C. A. de Groot, Department of Pharmacology, who participated in many of these experiments, Miss M. Persant Snoep for technical assistance, and Dr. A. C. Lendrum for his criticism.

Most of these experiments were done while I was working in the Department of Cytology and Experimental Histology (Director, Dr. P. J. Gaillard).

TECHNICAL PROCEDURES

Explants of the anterior lobe of the hypophysis and the peripheral part of the adrenal cortex of 1-week-old rats were cultured in separate containers on top of a coagulated medium from a human fetus diluted 10 times with Hanks' balanced saline solution. Twelve explants were cut from one anterior hypophysis. The cortical explants measured between 0.3 and 0.5 mm.

Every 3d day the explants were removed from the clot, washed in saline, and transferred to a freshly prepared medium.

After a culture period of 6 days the cortical explants were combined with fresh explants, with 6-day explants (*i.e.*, previously cultured for 6 days), and with 12-day explants of the anterior hypophysis. Some of these 12-day explants had been cultured in the presence of fragments of the median eminence, a fresh fragment being added each time the hypophyseal culture was transferred to a fresh medium. Explants of the adrenal cortex cultured without hypophysis or combined with renal tissue served as controls.

After a combined culture period of 3 days the combined cultures were fixed, and the medium was collected for corticosteroid estimations [Saffran and Schally method (*4*)]. The medium was pooled from 4 culture glasses, Maximow depression slides, in each of which 3 combined cultures had been grown. In this way, about 0.5 ml of medium was available for one estimation. The morphological changes in the adrenocortical cells elicited by the hypophysis and the amount of corticosteroids in the medium of the combined cultures, as compared with the amount in the controls, served as an indication of the corticotrophic activity.

To test the survival capability of the hypophyseal cultures the following preliminary experiment was tried: Six hypophyseal explants cultured for 3 weeks were transplanted to the sella turcica of each of six 3-month-old hypophysectomized rats belonging to the same strain as the original animals. Explants transplanted under the capsule of the kidney of hypophysectomized rats served as controls, as did normal rats and hypophysectomized rats without transplants. Five months after transplantation, the 5 surviving animals were killed. The corticotrophic activity of the transplanted cultures was assessed by measurement of the difference between the weight of the left adrenal, removed 10 days before the animal was killed, and the weight of the right adrenal at the time of death. Serial sections were made of both the sella turcica and the site of transplantation under the renal capsule.

RESULTS

The adrenal explants cultured alone (fig. 3) or in contact with explants of the kidney (figs. 1 and 2) showed an identical picture. The explants were encapsulated by outgrowing fibroblasts during the first few days of the culture period. The zona fasciculata degenerated and regeneration took place from the inner part of the pre-existing capsule and from the cells adjacent to the capsule. After 9 days, in most cultures, the degenerated areas had been replaced by cells indistinguishable from those normally found in the zona glomerulosa. Sometimes a few scattered fasciculata-like cells were seen.

The explants of the anterior hypophysis (fig. 4) showed little or no initial degeneration. After a culture period of a few days, they were encapsulated by epithelial cells. The granulation of the chromophil cells disappeared rapidly, and by the 6th day the culture consisted entirely of degranulated chromophobe cells. The presence of fragments of the median eminence in a joint culture had no influence on the morphology of the hypophyseal cells.

The presence of explants of the anterior hypophysis had a significant influence on the cortical cells, but this varied greatly in the different cultures. Thus, in some the adrenal cells showed a slightly decreased nuclear-cytoplasmic ratio and a coarse cytoplasm sometimes with small vacuoles, while in others there were numerous cells with large vacuoles enclosing a colloid-like material. Some of these cells with large vacuoles were obviously degenerate; they had ruptured and their nuclei showed clumping of the chromatin. The cells immediately adjacent to the pre-existing capsule were also modified by the presence of anterior hypophysis. This seemed most obvious in the adrenal cultures that had been combined with fresh explants of the anterior hypophysis (figs. 5 and 6)—the hypophysis explants that had been cultured separately for some time having apparently lost activity during this period. Nonetheless, several cultures combined with 12-day cultures of hypophysis showed pronounced changes (figs. 7 and 8).

No differences could be observed between the cortical explants combined with hypophyseal explants previously cultured for 12 days with or without fragments of the median eminence.

The corticosteriod estimations indicate that the morphological changes in the cortical cells elicited by the hypophyseal explants go hand in hand with an increased release of corticosteroids (table 1). Even the 12-day explants of the hypophysis stimulate the corticosteriod production. The median eminence had no significant influence on the corticotrophic activity of these cultures.

TABLE 1.—Corticotrophic activity of organ cultures of the anterior hypophysis*

Combinations	Number of estimates	Corticosteroid in γ per cc
I. Adrenal	13	3.8 ± 0.7
II. Adrenal + kidney	6	3.2 ± 0.7
III. Adrenal + hypophysis ^{0}†	12	8.7 ± 1.7
IV. Adrenal + hypophysis ^{6}	11	8.4 ± 1.0
V. Adrenal + hypophysis ^{12}	9	6.8 ± 1.3
VI. Adrenal + (hypophysis ^{12}+ median eminence)	9	8.8 ± 1.5

* Statistical significance: III > I and II ($P_2 < 0.002$); IV > I and II ($P_2 < 0.002$); V > I and II ($P_2 = 0.038$); III > V ($P_2 = 0.92$); VI > V ($P_2 = 0.89$).

† Hypophysis (0, 6, 12) means: explants of the hypophysis previously cultured for 0, 6, or 12 days.

Microscopically the sella turcica implants in hypophysectomized animals revealed remarkable changes in the hypophyseal culture. At the time of implantation the cultures consisted entirely of degranulated cells. Five months later, in 4 of the 5 surviving animals, there were vascularized masses of well-differentiated chromophil and chromophobe cells (figs. 9 and 10). The original cultures had increased in size and were surrounded by connective tissue showing no reaction to the transplant. On the other hand, the implants under the capsule of the kidney of hypophysectomized rats consisted entirely of undifferentiated cells (figs. 11 and 12). The weight of the adrenal had decreased, compared with the control group of normal rats, but loss of adrenal weight was greater in both the hypophysectomized animals without transplants and in the hypophysectomized animals with transplants under the renal capsule. Three of the 4 animals with viable transplants showed compensatory hypertrophy of the right adrenal after extirpation of the left adrenal.

DISCUSSION AND CONCLUSIONS

The results of this study show that organ cultures of the anterior hypophysis stimulate for some weeks, at least, a release of corticosteroids from adjacent cultures of the adrenal cortex. It seems unlikely that the increased amount of corticosteroids in the medium could be the release of corticosteroids already in the cortical cells at the time of explanting, because the cortical explants had been cultured for 6 days before they were combined with the explants of the anterior hypophysis. Also, during this period, the medium was refreshed twice and the cultures were washed in Hanks' solution each time. It seems much more likely that under the influence of the corticotrophic activity of the hypophyseal explants a real increased corticosteroid production had taken place in the cortical cells.

Loss of corticotrophic activity in the cultured hypophyses occurred over some weeks, and, as Petrovic (*5, 6*) and Martinovitch (*7*) have also noted, this varied from culture to culture. Petrovic regards embryo extract as being inimical to continued activity, and certainly our medium contained human fetal-brain extract. Another view is that hypophyseal tissue is fundamentally dependent on the products of the nearby hypothalamus. Guillemin (*8*) states that both tissue cultures and organ cultures of the anterior hypophysis lose their ACTH activity after a culture period of about 3 days, but this is reinitiated, or is considerably increased in amount and duration, by combined culture with hypothalamic tissue, or merely by the addition of hypothalamic extracts.

Unfortunately, we obtained no beneficial effects from the presence of adjacent culture of fragments from the median eminence. The hypothalamic factor Guillemin postulates may be less effective or nonexistent in our culture system, but our implants in the sella turcica seem in fact to favor such a factor, for not only did the degranulated chromophobic cells of the culture become chromophilic but also the adrenals, as the target organ, suggested that these implants were producing corticotrophic hormone.

Study of the granules has shown that there is a failure of the staining reaction before the functional failure becomes obvious. It has, of course, been tempting to think that the granules with their different affinities for various dyes are the repository of prepared hormone, and it is interesting that Petrovic (*6*) found that when the granules disappear the ultrastructure of the cells is still perfectly normal and, except for a decreased number of granules, indistinguishable from normal hypophyseal cells. It is not yet possible to say whether the gradual degranulation of the *in vitro* cultures is evidence of a diminishing health in the epithelial cells or of a fast turnover where any hormone formed is at once expelled.

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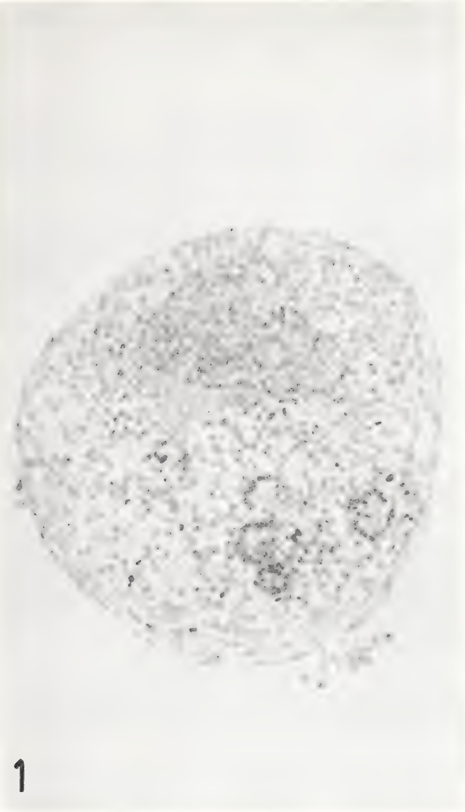
Plate 14

Figure 1.—Explant of the peripheral part of adrenal cortex cultured for 6 days and then combined with a fresh explant of kidney. *Upper half* shows the cortical tissue; *lower half*: the renal tissue. × 180

Figure 2.—Same section as figure 1, high magnification of the subcapsular cortical tissue. These cells have little cytoplasm. × 1,200

Figure 3.—Explant of the peripheral part of adrenal cortex after cultivation for 9 days. × 160

Figure 4.—Explant of the anterior hypophysis after cultivation for 15 days. × 230



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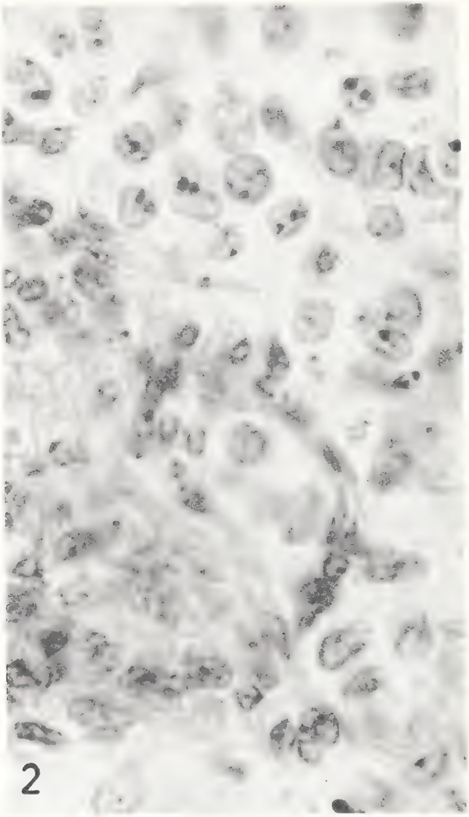

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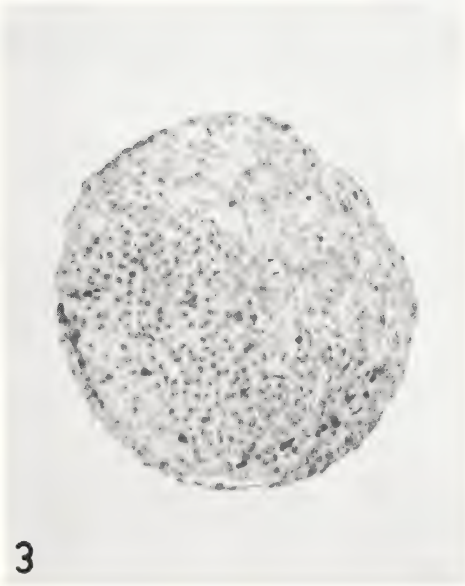

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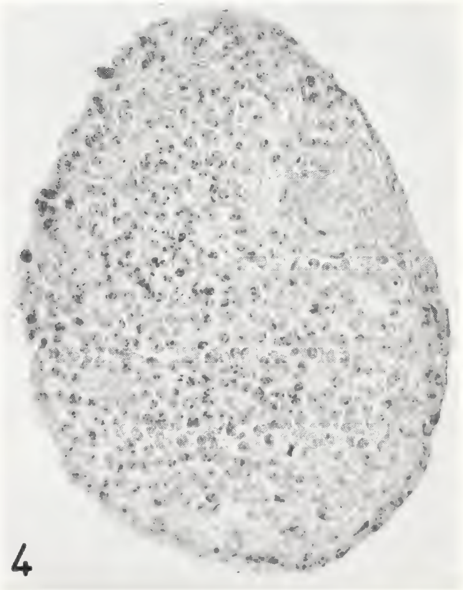

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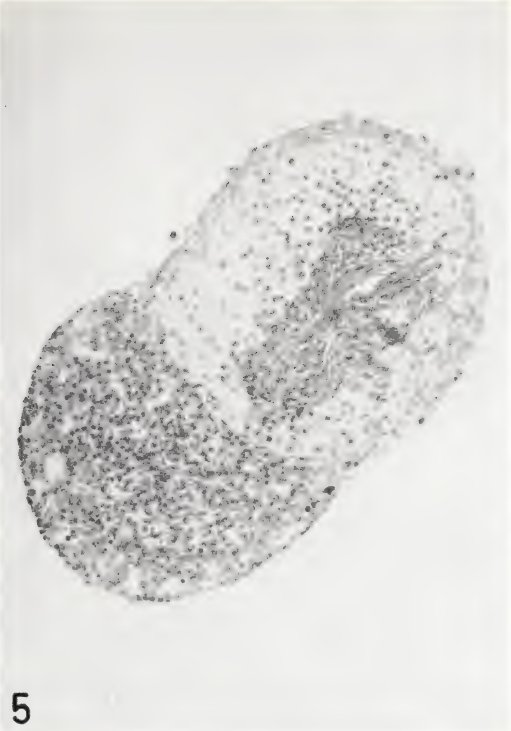
Plate 15

Figure 5.—Explant of the peripheral part of the adrenal cortex cultured for 6 days and then combined with a fresh explant of the anterior hypophysis. *Upper half:* adrenal cortex with the pre-existing capsule in *center; lower half:* the hypophysis. × 160

Figure 6.—Same section as figure 5, high magnification of the subcapsular area of adrenal cortex. There is a marked decrease of the nuclear-cytoplasmic ratio in comparison with controls. Cytoplasm is somewhat coarse. × 1,200

Figure 7.—Explant of the peripheral part of adrenal cortex cultured for 6 days and then combined with an explant of the anterior hypophysis previously cultured for 12 days. *Upper half:* adrenal cortex; *lower half:* hypophysis. × 150

Figure 8.—Same section as figure 7, high magnification of the subcapsular area of adrenal cortex. Nuclear-cytoplasmic ratio has only slightly decreased in this region. × 1,150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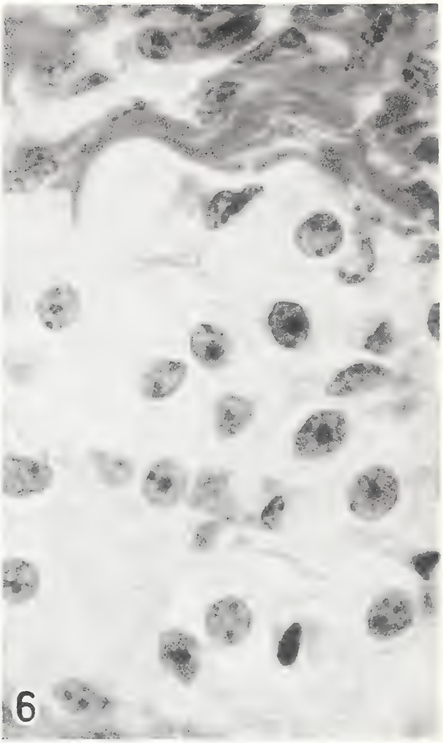
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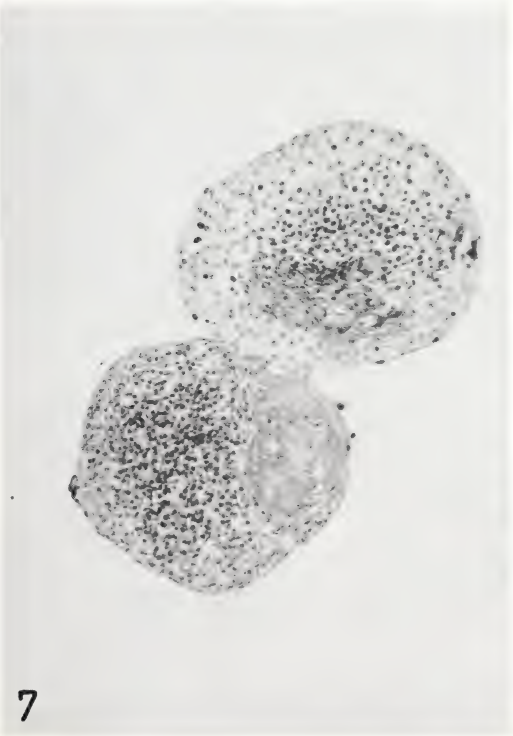
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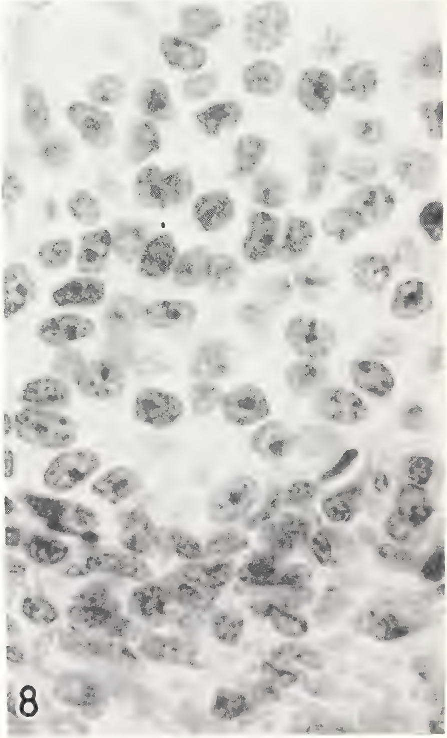
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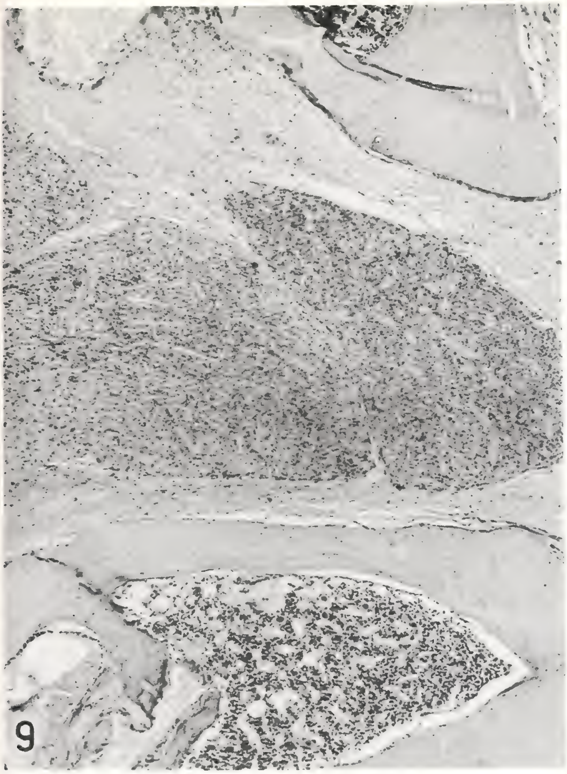
Plate 16

Figure 9.—Explants of anterior hypophysis cultured for 3 weeks and then transplanted to the sella turcica of a hypophysectomized rat. Transplanted cultures 5 months after transplantation. × 56

Figure 10.—Same section as figure 9, high magnification of the transplants, showing well-differentiated chromophil and chromophobe cells. × 140

Figure 11.—Fresh explant of anterior hypophysis transplanted under capsule of the kidney of a hypophysectomized rat, 5 months after transplantation. × 56

Figure 12.—Same section as figure 11, high magnification. Transplant consists entirely of undifferentiated cells. × 350



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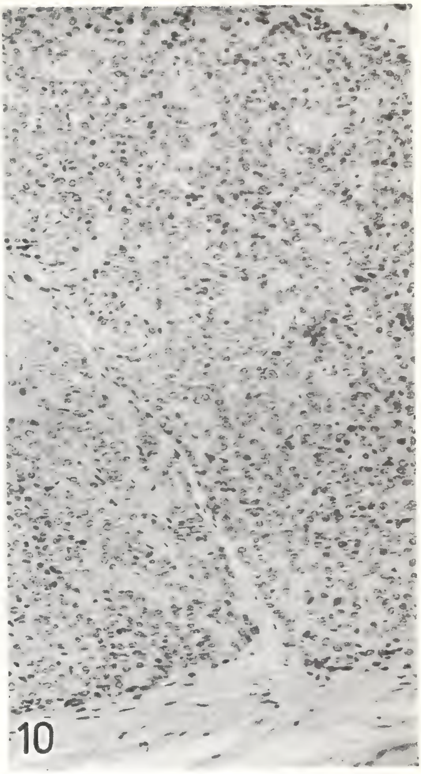

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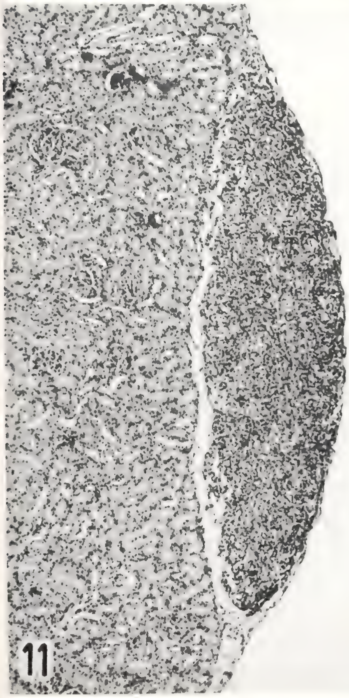

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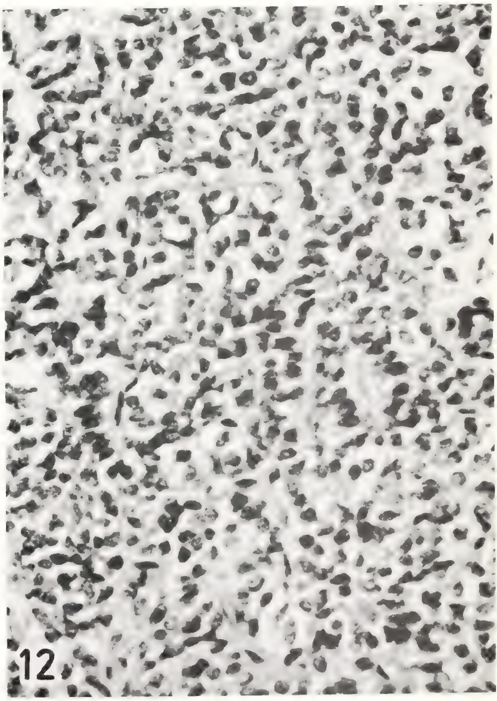

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Dr. Schaberg's discussion of his outstanding investigations on the adrenal cortex (*1*) and its relationship to the anterior hypophysis (*2–5*) has been a source of constant encouragement to my personal belief that organ culture is a potent tool for analyzing physiological relations (*6*). Present available evidence suggests rather clearly that when tissues are cultivated as isolated cells they lose most, if not all, of their functional ability to produce specialized products such as hormones (*7–10*).

However, one must not assume that organ explants which histologically resemble normal *in vivo* structures are functioning in a normal manner. Dr. Schaberg has based his analysis of corticotrophin production on two facts: 1) the cytological transformation of zona glomerulosa-like cells to vacuolated cells typical of the zona fasiculata and 2) the production of corticosteroids by adrenal explants as assayed by the Saffran and Schally (*11*) method. Since this technique has been criticized as relatively insensitive (*12*) and, more importantly, as responding to nonspecific protein or protein-bound factors (*13, 14*), perhaps more sensitive methods such as the adrenal ascorbic acid-depletion method of Sayers *et al.* (*15, 16*) would be justified as corroborative evidence. In addition, the cytological transformation of the adrenal *in vitro* would lend itself to histochemical analysis. Although the available histochemical procedures for demonstration of ketosteroids are poor at best, they would provide some insight into the problem. For example, a battery of reactions has been proposed which correlates sudanophilia, birefringence, acetone solubility, fluorescence, Schiff's reaction, and the dinitrophenylhydrazine reaction with adrenocorticosteroids (*17*). These procedures would establish if the adrenal vacuoles represent lipide deposits. Furthermore, visualization of steroid 3-β-ol dehydrogenase activity in the adrenal by the method of Levy *et al.* (*18*) would permit correlation with their findings *in vivo*, namely, localization of this enzyme at sites of corticoid synthesis.

Of more concern to the endocrinologists is the large body of evidence which suggests that the anterior hypophysis, transplanted to the kidney capsule or the temporal lobe, is capable of secreting luteotrophin and perhaps somatotrophin (*19*) with a concomitant loss in gonadotrophin, thyrotrophin, and corticotrophin secretion (*20–25*). As Dr. Schaberg pointed out, hypothalamic factors are required to stimulate corticotrophin production (*26*), while luteotrophin appears to be normally under an inhibitory influence from the central nervous system (*27*). Perhaps the corticotrophin release that Dr. Schaberg has demonstrated represents the residual production and/or release of this trophic hormone. Hertz (*28*) found it necessary to use multiple homografts of the anterior hypophysis to elicit an adrenal response to amphenone. Possibly the human fetal-brain extract which Dr. Schaberg employed for his media may provide a transitory corticotrophin-releasing factor. Another, and more intriguing, possibility is that although the hypothalamus is normally stimulatory to corticotrophin release, systemic factors may inhibit the release of this hormone so that the gland *in vitro* is once again permitted to be secretory.

Our corresponding interest in the problem of trophic hormone production *in vitro* stimulated us (*29*) to investigate the production of luteotrophin by organ explants. The choice of this specific hormone was reasonable from the available data on the transplanted hypophysis. Rat anterior hypophysis was cultivated on a completely defined medium, either medium 199 (*30*) or a modification (*31*) of Trowell's (*32*) medium T-8 with added insulin. Explants were cultivated in air or gassed with 95 percent oxygen and 5 percent carbon dioxide. Assay of the medium by the pigeon intradermal method (*33*) strikingly demonstrated that the production of luteotrophin per hypophysis per day equaled or exceeded the normal content of the intact anterior hypophysis when the gland was obtained from a resting breeder female. Lactating females produced correspondingly higher amounts while weanling females released less. Recently, Pasteels (*34*) corroborated the *in vitro* production of prolactin and went on to show in a pilot study that the addition of hypothalamic explants inhibited the release of this hormone (*35*).

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[1] Supported by research grant 6976 (C-2) from the National Institutes of Health, Public Health Service.

Everett (*22, 23*) and Sanders and Rennels (*36*) have reported that the predominant cell present in pituitaries transplanted to the kidney capsule is the acidophil. Purves (*37*) characterized the carminophilic subtype of acidophils to be the source of luteotrophin in most species, man being the prime exception. Of interest to this discussion is the fact that the cellular source of corticotrophin has not been definitely established. While most investigators have associated the basophils with corticotrophin production (*37*), Farquhar (*38*) has proposed the chromophobe as the cellular source of this trophic hormone. On the other hand, Finerty *et al.* (*39*) have provided evidence for the acidophil as the site of its production. Cytological studies in our laboratory of the anterior hypophysis cultivated *in vitro* for 7 days (*40*) suggest that the main residual cell is a degranulated acidophil, whereas most of the beta cells degenerate rapidly and only a few delta cells remain. On the other hand, Dr. Schaberg has reported that his organ cultures of the anterior hypophysis "consist entirely of degranulated chromophobe cells." In view of the considerable difficulty in identifying cell types of freshly fixed anterior hypophysis and the concomitant confusion in terminology, extreme caution should be taken in any interpretation of cellular changes *in vitro*. This becomes even more important when it is realized that the anterior hypophysis is very sensitive to manipulation during which changes in morphology and stainability may result.

Finally, it should be re-emphasized that the organ culture technique provides an excellent system for the study of endocrine relationships despite the attendant pitfalls. The pioneering work in this area by Dr. Schaberg is a distinguished example of the gains possible.

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STUDIES OF FUNCTION IN ORGAN CULTURE: SUMMARY, CORRELATION, AND SPECULATION[1]

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It is a great pleasure to be with you and to contribute to your first symposium concerning "Studies of Development, Function, and Disease in Organ Culture." You know that I am expected to summarize, correlate, and also speculate about the work presented during this session on Function. This is not easy and often not rewarding, but I am happy that I can make some of my visions and convictions known to you.

First, Dr. Franks drew our attention to a number of fundamental facts concerning the organ-culture method. His remarks should be fully recognized by everyone applying this method. In particular, I would like to stress his point that the explants must be small enough to allow diffusion of nutrients, oxygen, and waste products of metabolism, but as to the actual optimal size of the fragments I would add that various types of tissue may behave differently in this respect. At least in our hands some tissues, such as most of the endocrine glands, must be cut into very small fragments to obtain an optimal result, while other tissues, such as periosteal fibroblasts, chick-embryo heart, human fetal skin, and so forth, can be cultured effectively in much larger fragments. For some tissues, however, the situation seems more complicated, the size of the fragments being relatively less important. For fetal *human* ovarian cortex, for instance (figs. 1, 2, 3, and 4), the fragments first show an intensive degeneration involving practically all parenchymal cells whether they are in the center or periphery of the explants. At the same time, the germinal epithelium, occurring at the surface of the ovary as well as the major part of connective tissue in the interior of the explants, remains intact. Then, within a few days, the germinal epithelium gives rise to the formation of a complete capsule from which subsequently a new and characteristic ovarian cortical structure with oöcytes and primordial follicles regenerates (*1–4*).

With parathyroid-gland tissue another type of reaction can be observed. First a rather severe deterioration of the superficial tissue occurs. Then, after 2 or 3 days, at quite some distance from the edge of the fragment, an epithelial membrane appears bordering the healthy and the unhealthy

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part of the fragment (fig. 5). Subsequently, a rather fast growth of the tiny healthy center occurs, which soon reaches a definitely larger size than the original fragment (fig. 6). Both in the ovary and the parathyroid tissue no, or hardly any, outgrowth occurs and the organotypical tissue can be kept in culture for periods varying from 1 to 2 months (*5*, *6*).

Still another behavior has been demonstrated by Dr. Zaaijer (*7*) concerning the culture of human phalanges. If the phalanges are taken from embryos that are older than 9 weeks, only the rudiments which became surrounded by a very loose reticular type of connective tissue and by an encapsulating epithelium showed a really organotypical development.

On the basis of Dr. Franks' observations, and considering the few examples I have just given, I believe it is justified to conclude that explants, within certain limits of size, may indeed behave quite differently.

Concerning the very process of dissection itself, I fully agree with Dr. Franks' remarks. In the course of time one learns to handle each specific type of tissue and one has to adapt the technical procedure to the tissue to be investigated. However, in all cases one must be extremely careful and must avoid long-lasting procedures, the drying out of the bathing fluid, and the occurrence of too excessive temperature variations. In long-term experiments damage caused by such defective technical procedures might perhaps be overcome, but certainly in short-term experiments they can be the cause of very inconsistent results.

Of particular importance also is Dr. Franks' statement concerning the "failure" of outgrowing disorganized sheets of cells to function. This is really a fact which should be kept in mind, although it does not imply that the outgrowing cells have automatically lost their potentiality to function. Re-establishment of adequate cellular interrelationships, including the correct composition of the microenvironment, may lead to a resumption of functional activities. It must be stated, however, that after a really prolonged cultivation of disorganized and fastly dividing cells such a capacity may obviously become lost. On the other hand, there are examples where some functional activities appear to go on in sheets or even in isolated cells. One example I recall is the formation of argentophil fibers in the outgrowths of fibroblastic elements. In this connection, also, Hilfer's observations (*8*) on isolated thyroid cells may be of interest. He demonstrated that spread cells of the chicken-embryo thyroid first lose their periodic acid-Schiff-positive granules, but after about 7 days of culture the number of granules increases again. Also such cells still contain mono- and diiodothyronine. The faculty to produce thyroxine, however, had declined.

In regard to the disputed balance between proliferation and cell function, I believe that for some tissues proliferation itself is an intrinsic part of its functioning, such as blood cell-forming tissues, testes, etc. In my opinion, the question is whether, apart from this, cells involved in the process of reproduction are, at the same time, able to use their specific machinery for other functional purposes, such as fibrillar and secretory activities. Quite a few arguments have accumulated, which have led us to think that this is not the case. One should realize, however, that a tissue is built up of many cells and that, as a rule, they do not divide simultaneously. In fact, a division of labor is apparent in many tissues.

If it is true that dividing cells do not show other functions at the same time, one may ask whether by simply suppressing the growth of such cells automatically conditions are created which cause them to function. In this respect, I recall one of our own contributions using a strain of periosteal fibroblasts cultured in media containing press juices of embryos of different age. In these experiments the growth (cell division and cell migration) was not only quite similar in explants cultured in a medium containing a press juice of 4-day embryos as compared with those cultured in medium with a press juice of 15- to 18-day embryos, but both had completely stopped growing after 4 or 5 days of cultivation. Nevertheless, in the former explants no prebone became apparent in the centers of the cultures, whereas in the latter group a significant development of such a differentiated tissue was observed.

Concerning Dr. Franks' investigation on prostate tissue, I believe the role of insulin inducing quite an irregular proliferation of the epithelium is most interesting. Also Dr. Ambrose has told us about some of the influence of insulin on the faculty of lymphoid tissue to form antibodies. In this connection I may also call your attention to some of Dr. Prop's experiments (*9*) performed in the laboratory of the Netherlands Cancer Institute at Amsterdam. This author reported that the mouse mammary gland does not respond to added prolactin unless insulin is present in the medium. Also, in our own studies on the effect of parathyroid extract on cultured long-bone rudiments, an effect of insulin became apparent since it prevented some parts of the rudiments from reacting to parathyroid extract. Therefore, without going into detail, I believe it is perhaps a bit early to consider such effects of insulin to be organ-specific. Dr. Franks' observation that after he transferred the insulin-treated explants into a serum-containing medium the hyperplastic cells disappeared again and a normal structure was re-established is most interesting.

From all this it is not too difficult to speculate a little about future developments, especially in the use of organ cultures of endocrine glands or target tissues for hormones and vitamins. In this connection I think the time has come when important contributions can be made concerning the molecular biology of the action of hormones and vitamins on isolated model systems. Microbiochemistry, cytochemistry, and histochemistry (also on ultrathin sections), as well as electron microscopy and tracer techniques, are some of the means by which a penetration toward the metabolic properties of small amounts of tissue can be realized. For example, we have been studying the influence of parathyroid extract on the mouse-embryo radius *in vitro* (*10*). Experiments with C^{14}-glycine revealed that after some 6 hours of cultivation in the presence of 0.1 to 2.5 IU of parathyroid extract (PTE) the glycine-binding capacity of the osteoblasts was significantly diminished (table 1). Also with autoradiography, after addition of tritiated glycine an early defect in the functioning of the osteoblasts could be established. Further biochemical (*11*) and histochemical (*12*) studies revealed that as far as enzyme activities are concerned, the hexose monophosphate shunt is very active in normal bone, but in PTE-treated rudiments TPN-dependent steps of the shunt as well as the Krebs cycle are inhibited in this experimentally produced hyperparathyroidism *in vitro.* Without claiming that experiments with organ cultures will lead to a final solution of our problems, I believe that without such model systems one can hardly study hormone effects without other hormones and feedback mechanisms interfering with our results.

TABLE 1

Controls (left-hand radii)		Parathyroid extract 2½ IU per cm^3 (paired right-hand radii)		
Number of explants pooled	Counts per 5 minutes (shafts only)	Number of explants pooled	Counts per 5 minutes (shafts only)	Percent of control values
3	2511	3	1671	67
3	4438	3	2451	55
3	4510	3	2956	65
3	3327	3	2208	66
3	2918	3	1568	54
				61±6% (P = 0.5%)

The advantage of the use of electron microscopy can be demonstrated in the following example: Mrs. Bakker-Sauer (*13*), of our department, described some years ago that in explants of the thyroid gland of 8-day chick embryos large vacuoles, or "antra," appeared in the presence of certain concentrations of thyroid-stimulating hormone (figs. 7 and 8) prior to the stimulation of colloid formation. These vacuoles were interpreted as being intracellular until examination under the electron microscope clearly revealed that the holes had developed from preexistent submicroscopical intercellular lumina (*14*, *15*). At the same time in response to TSH the embryonic thyroid cells matured by development of its endoplasmic reticulum. Moreover, biochemical investigation revealed that most likely the formation of the "antra" went hand in hand with the appearance of an increased amount of a proteolytic enzyme.

In the next paper in this session, Dr. McGowan confronted us with quite a different field of investigation, namely, the study of herpes simplex-infected organ cultures of the skin. He succeeded in culturing full-thickness human fetal skin in such a way, and during such a long period, that he was able to produce a real virus disease *in vitro.* I think his work is a beautiful demonstration of what can be done with the help of organ-culture methods to unravel some problems of this kind without the interferences of circulatory factors and control mechanisms of the nervous system.

For the study of viral and other infectious agents, organ-culture methods can therefore be expected to be of great help. However, for another group of diseases, especially during the last 10 or 15 years, quite interesting results have become available, *e.g.,* Dr. Fell's beautiful experiments (*16*, *17*) on the effects of excess vitamin A on skin and long bones. Also our own work on the effect of hyperparathyroid conditions on embryonic bone rudiments clearly indicates that a real bone disease with defective osteoblastic function and osteoclastic bone resorption can easily be obtained and studied.

In problems of malignancy the time has come for fruitful studies: for instance, Dr. Lasnitzki's (*18*) and Dr. Franks' work on prostate tissue concerning problems of tumorigenesis and Dr. Wolff's study (*19*) on invasive growth, using combined organ cultures of tumor tissue and mesonephros.

In the study of viral, metabolic and tumor diseases, organ-culture methods can be of great help and it is not difficult to foresee much more work in this direction in the years to come. I think it is fair to state that *organ* cultures can serve as ideal models for the study of processes concerning disease.

In the third paper, Dr. Ambrose has confronted us with another type of work that can be performed with the aid of organ cultures. He applied an adapted organ-culture method to the study of antibody production by lymph-node explants. Like Dr. Franks and Dr. McGowan he was also faced with problems concerning the role of the culture medium for preserving the functioning of the tissue fragments. The data presented by Dr. Ambrose speak for themselves and I think that it would be of great value to correlate his findings with the cytological and histological characteristics of the lymph-node explants during the period of cultivation. Personally I was very interested in what Dr. Ambrose told us about the significance of adding hydrocortisone and insulin. The results obtained with chloramphenicol which he proved interfered with the metabolic events, especially in the early part of the secondary response, demonstrate what can be done with isolated lymph-node material.

In regard to the role of the cellular constituents in the production of antibodies, Dr. Robineaux (*20*) in Paris quite recently succeeded in the culture of spleen tissue, according to a modified Rose method, by placing slices of tissue in between two cellophane membranes in a fluid culture medium. In this way he obtained a really beautiful differentiation of numerous plasmocytes, which in relation to antibody formation could be quite important.

Finally Dr. Schaberg's paper illustrated the significance of the organ-culture method in studying interrelationships of endocrine systems. His findings clearly demonstrate that it is not always necessary to keep the original structure of the organ intact for obtaining the information which we want. On the contrary, the transformation of the adrenocortical explants into organoids mainly composed of glomerulosa-like cells has enabled him to study the reaction potentialities of these cells under the special circumstances of the method. With the anterior hypophysis, his findings clearly indicate that although rather considerable histological changes occurred in the anterior hypophysis tissue such cultures still are able to release substances which like ACTH exert a specific corticotrophic activity.

It is of interest to realize that after transplantation of the degranulated anterior hypophysis tissue into the sella turcica a characteristic histological aspect of the tissue and a normal functioning of the transplant could be observed. This showed that the potency to develop a normal histological and functional picture had not been lost.

In the past there has often been misunderstanding as to what experiments with isolated organs or parts of organs could really reveal. I feel however that the present meeting has shown that organ-culture methods can be of great help in solving our problems. On the other hand, these methods are neither worse nor better than any other method which can be used. One should realize that results obtained with it can never be immediately transferred to what is called "the normal situation." It is, however, great fun to do this kind of research and to find that the reactions of an isolated model are indeed of great help in the understanding of the complicated machinery occurring in the living individual.

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PLATES

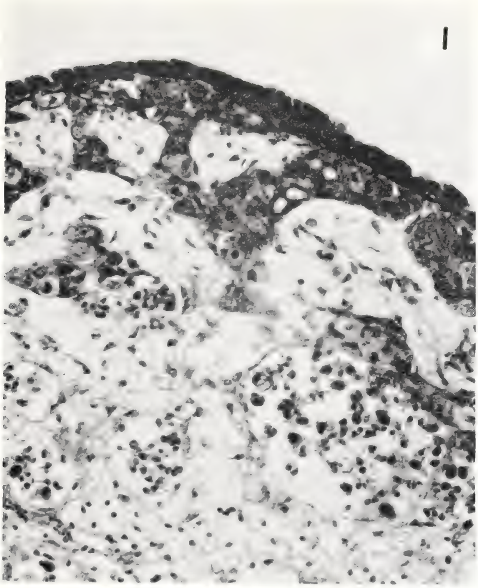
PLATE 17

FIGURE 1.—Explant from ovarian cortex of a 20-week-old human fetus after 4 days of cultivation. Parenchyma in the interior shows many degeneration phenomena. In the meantime, the germinal epithelium encapsulated the fragment. × 245

FIGURE 2.—Explant from ovarian cortex of a 26-week-old human fetus after 10 days of cultivation. Regenerating parenchymal cords and tubules have penetrated toward the interior of fragment, which has been cleared from cellular debris. The center is now composed of a reticular connective tissue. × 120

FIGURE 3.—Explant from ovarian cortex of a 20-week-old human fetus cultivated for a period of 10 to 11 days. *Note* increased number of anastomosing cords of parenchymal cells. A number of young oöcytes has been formed. × 245

FIGURE 4.—Explant from a part of the ovarian cortex of a 16-week-old human fetus after 12 days of cultivation. Number of cords has increased considerably and a fairly normal cortical structure with some primordial follicles has developed. × 280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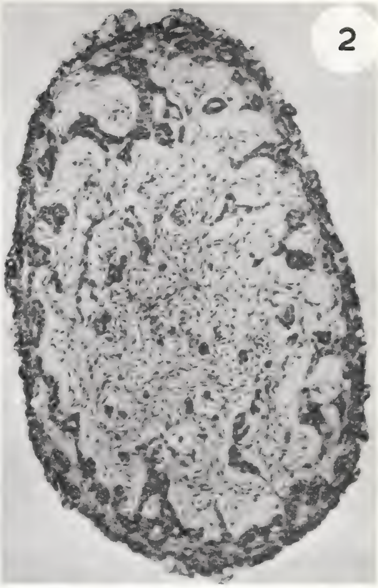
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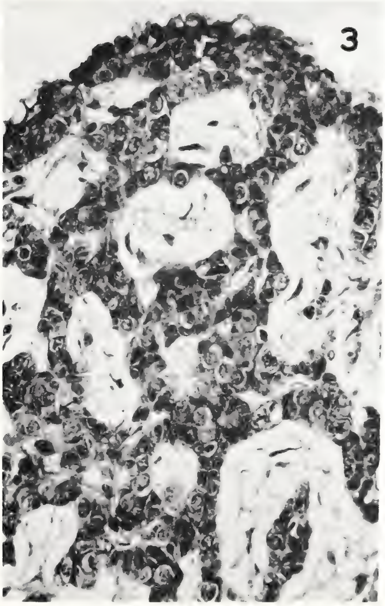
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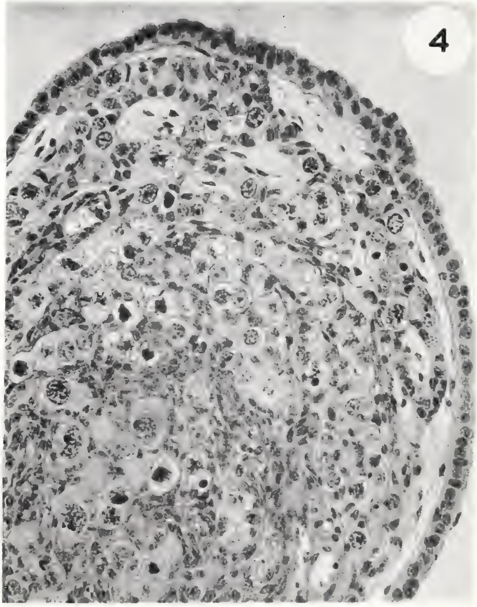
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Plate 18

Figure 5.—Fragment of parathyroid gland of a human newborn, cultivated for 10 days. In *center* some nests of clear cells are visible. Explant is surrounded by a layer of parenchymal cells. × 160

Figure 6.—Fragment of parathyroid gland of a human newborn, cultivated for 26 days. *Note* size of the fragment and the histotypical structure of the tissue. × 160

Figure 7.—Electron micrograph of thyroid-gland tissue of noncultivated 8½-day chick embryo. *Note* small intercellular lumen with microvilli and desmosomes. × 12,000

Figure 8.—Electron micrograph of an explanted thyroid gland of 8½-day chick embryo, 2½ hours after onset of the cultivation period in the presence of 2½ IU of TSH per ml of culture medium. *Note* enlargement of the intercellular lumen. Microvilli have disappeared. × 12,000

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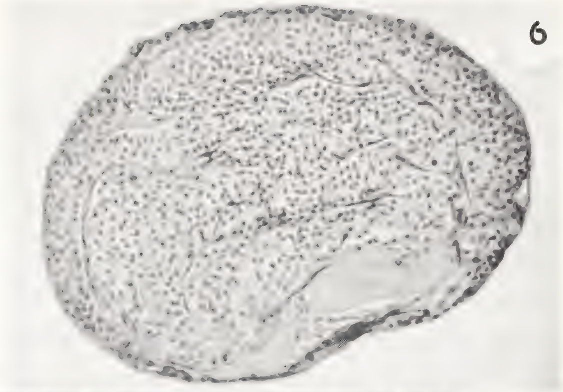
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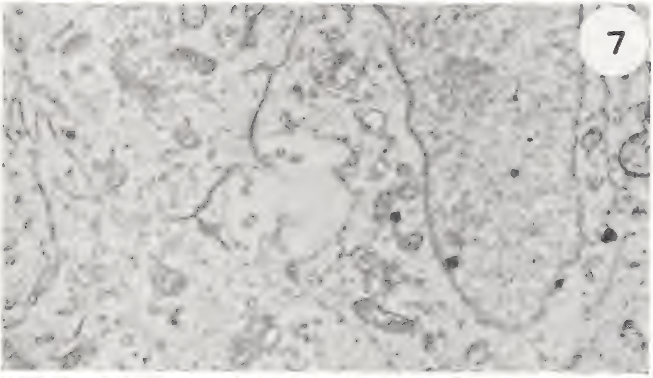
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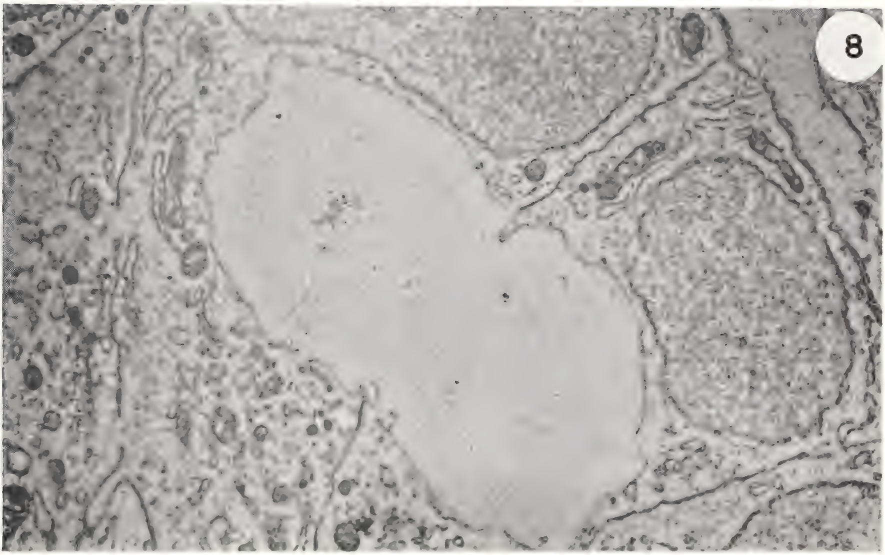
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Session III

Studies of Disease in Organ Culture

Chairman: Frederik B. Bang, *Johns Hopkins University School of Hygiene, Baltimore, Maryland*

A METHOD FOR THE COMPARISON OF THE FATE OF INTRAVASCULAR TUMOR-CELL EMBOLI *IN VIVO* AND IN ORGAN CULTURE [1,2]

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SUMMARY

When tissue-culture methods have been used in the study of invasion, the relationship between events seen *in vitro* and invasion observed in the intact animal has been uncertain. Usually a fragment of previously normal tissue is confronted abruptly with a comparatively massive fragment of cancer tissue. The "site of invasion" examined is usually the zone where the outgrowths from the 2 explants meet, and the cellular interaction is observed out of the context of the intact animal. Some of these difficulties are being resolved with a technique under development in which tumor cells are brought in contact with normal tissues *in vivo* by intravenous inoculations, and the organs of the host thus inoculated are removed for study, for culture, or for transplantation to other animals. We have inoculated suspensions of tumor cells intravenously in the chorio-allantois of 11-day chick embryos. The fate of tumor cells in any organ is compared in three ways. The embryonic organ is removed shortly after inoculation, cut into fragments, and the fate of tumor emboli contained in the organ is studied in organ culture and as transplants to the chorio-allantois of normal embryos. The fate of these emboli is compared 1 week later with that observed in intact 18-day embryos. Preliminary data have been obtained with cells of rodent ascites tumors and tissue-culture lines.—NCI Monogr. No. 11: 157–195, 1963.

AFTER INOCULATION of a suspension of tumor cells in a vein of the chorio-allantoic membrane of the chick, tumor-cell emboli lodge in a variety of sites in the embryo itself and in the extraembryonic membranes. The fate of these emboli in the tissues of the intact chick can be compared with observations made after transplantation or culture of selected tissues bearing such emboli.

One stimulus for the development of this method of study has been the limitations of the techniques previously employed. A method used for several decades (*1*), the simplest tissue-culture technique for the study of tumor invasion, is to place a piece of normal tissue and tumor tissue side by side in a plasma clot. Phenomena of cellular interaction have been

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observed where the outgrowths of normal tissue and cancer tissue met. Events occurring within the substance of the normal explant were usually not observed. The progressive infiltration and replacement of cells of the normal outgrowth by the growing tumor cells have usually been interpreted as an example of malignancy.

A second limitation of such methods is suggested by the fact that the initial masses of cancer tissue and normal tissue are approximately the same size, *i.e.*, the tumor is relatively massive. In addition, such cultures are usually examined microscopically from either the top or the bottom. Examination in this way precludes the possibility of any consideration of the role that the gradients of metabolites may play from the fluid-bathed surface of the culture into the depth of its many cell layers. Experiments in sponge-matrix tissue-culture systems suggest that the influence of these gradients in the determination of the outcome of an interacting system of tumor and normal tissue is of some consequence (*2*). Finally, none of the systems brings the tumor cells in contact with normal tissues by the physiologic or normal anatomic routes, and none permits a ready correlation with events observed in the intact animal.

The research of Professor Wolff and his associates represents outstanding progress in eliminating some of the undesirable factors mentioned. In his method, chick mesonephros is the living host in combinations with minute explants of tumor. The mesonephros does not grow out to any extent to meet the tumor, since an agar surface is used to support the explants rather than glass or a plasma clot. Dr. Wolff's illustrations of the invasive replacement of mesonephros by several kinds of cancer cells are very impressive (*3*).

The research conducted by Schaberg and that of Gaillard (*4*) considered the question of a better quantitative balance between the size of the tumor and the host tissue. Fragments of embryonic intestine that produce "epithelial balloons" or "intestinal sacs" in organ culture were inoculated by placement of minute tumor fragments into the substance of the intestinal organism. The inoculation of the tumor fragment within the substance of the normal tissue is a major technical variation to be contrasted with systems in which the tumor cells are placed in contact with the surface of a normal tissue. The entire pattern of interaction and replacement of the normal tissue by tumor tissue may be modified, depending on whether the tumor explant is within the substance of the normal tissue or on its surface. When tumor cells are placed on the surface of a piece of normal tissue, the way in which they replace normal tissue may be by surface overgrowth of tumor cells and attrition of the normal tissue below the surface. Destructive growth by tumor cells from within an organ fragment may involve entirely different intercellular events.

The systems of Moscona, in which dispersed cells come together and cells of differing nature take cortical or medullary positions in the newly formed mass, may prove especially useful in relating the role of metabolic gradients to invasive behavior (*5*). Even in this situation matters are complex, for we are concerned not only with gradients of metabolites but at the very least with cellular affinities and disaffinities as well as with other events on the cell surfaces.

The work reported here is a continuation of previous studies on the spread of cancer in which local cellular interaction *in vivo* and *in vitro* is emphasized. The tissue-culture studies have involved the use of a three-dimensional matrix of cellulose sponge. In this system explants of parenchymal tissues retain their normal histiotypic appearance, and the architecture of the outgrowth often consists of recognizable structures such as glands, cords, and branching capillaries. Using this system with combinations of cell lines (*e.g.*, HeLa) as the cancer and a variety of normal tissues of human and chick embryonic origin, we have observed that the abundance and the spatial orientation of fibroblasts are important in the local spread of cancer (*6*). Maximal spread was observed when fibroblasts are pointed in the direction of tumor growth. Infiltrative growth is least when fibroblasts and their fibrillary products are arranged parallel to the growing front of tumor cells.

Interaction between normal tissues and cancer tissues *in vivo* has been observed on the chorio-allantoic membrane with the use of the "companion-host" system. This is feasible since a variety of tissues, both normal and cancerous, are accepted by the chorio-allantois. In the experimental system transplants were placed in contact with each other on the chorio-allantois of 8- or 9-day embryos, and the eggs were opened on the 18th day of incubation. In some combinations, *e.g.*, Walker carcinosarcoma and chick embryonic liver, both transplants took and, in addition, there was massive tumor growth with extensive infiltrative replacement of the normal tissue by the cancer cells (*7*).

Because the Walker tumor grows rapidly on the chorio-allantois and exhibits a remarkable ability to invade and replace several chick embryonic tissues, we have wondered for some time whether the tumor would show a similar striking affinity for liver and other parenchymal organs when introduced by the intravenous route. The literature relating to the growth of intravenous transplants of tumor cells is not particularly encouraging. In the hands of one group, the best results were obtained with rapidly growing mouse sarcomas and these presented a number of takes below 20 percent (*8*). In addition, the techniques for intravenous inoculation previously described have seemed formidable.

We were encouraged to undertake such experiments late in 1961 after reading a study by Dr. R. Kent in which suspensions of thorotrast particles were injected intravenously into the embryonated egg to study the reticuloendothelial system (*9*). We inoculated 11-day embryos intravenously in the chorio-allantois using essentially the technique described by Dr. Kent. The inoculum consisted of a small volume of fresh tumor ascitic fluid obtained from rats bearing the Walker carcinosarcoma 256. The eggs were opened 1 week later, on the 18th day of incubation, and most of the survivors showed multiple foci of tumor growth in various embryonic tissues as well as a stippling of the chorio-allantoic membrane with many minute tumors (*10*).

The experiment was readily repeatable and equally good growth was obtained in the embryo and its membranes when another rat ascites tumor, the Yoshida hepatoma 7974, was inoculated. Of further interest was the finding that the architecture of the nodules produced on the chorio-allantois was distinct for each type of suspension. The tumors produced by the hepatoma were lobular, compact, and clearly delineated from the surrounding connective tissue. The growths produced by the Walker tumor were dense in their centers, but their margins were indistinct, as numerous single tumor cells infiltrated the surrounding connective tissue. From these preliminary studies, two lines of inquiry have emerged. The appearance of minute discrete tumors in a disseminated pattern suggests that an intravascular plating out of cells has occurred. Under consideration is the possibility that some of the isolated nodules of tumor may be derived from single cells. We are interested in the microscopic events that can be observed early in the life of these intravascular transplants shortly after inoculation.

The other line of inquiry, the one with which we are concerned in this report, has as its goal the development and refinement of a system for studying the fate of intravascular tumor-cell emboli. Our interest includes tumors that may grow extremely well in the embryonated egg and those that may not, including human tumors.

The importance of this last point is one that is readily appreciated. If we are to make more intelligent comparisons of the phenomena of malignancy as expressed by transplantable animal tumors, by experimentally induced cancers in animals, by tissue-culture cell lines, and by clinical cancer in man, we must have better biologic systems in which phenomena of malignancy can be examined. "Tumor virulence" has been studied with the use of the anterior chamber of the eye of the guinea pig, the cheek pouch of the hamster, the irradiated or cortisone-conditioned rat, and tissue-culture systems. The methods to be described may prove useful additions to existing techniques for a comparison of differing tumor cells in a context where the cells express some of their qualities of malignancy.

METHODS

Intravenous inoculation.—Eleven-day embryonated eggs were candled and a rectangle was drawn over a straight segment of vein. (The rectangle usually measured 8 mm by 16 mm.) The direction of blood flow in the underlying vein could be recognized since the blood flow was from small veins into larger ones. This was indicated on the shell with an arrow. Using an abrasive wheel on a dental motor, we cut a window through the shell along the lines of the rectangle without tearing the shell membrane. The shell was gently lifted off, exposing the opaque, white shell membrane below. The vein, not seen at this stage, was made visible by spreading mineral oil with a brush over the shell membrane. As soon as the vein was visualized (a matter of a few seconds) 0.025 to 0.05 ml of cell suspension was inoculated into the vein in the direction of blood flow with a ¼ ml tuberculin syringe fitted with a 27-gauge disposable needle. One or 2 layers of molten paraffin were applied immediately after inoculation to the entire area of the exposed shell membrane and adjacent shell. The eggs were returned to the incubator. We then soaked a piece of moistened Gelfoam sponge in some of the ascitic-fluid suspension and fixed this piece of sponge in 10 percent formalin for a microscopic representation of the donor fluid. The sponge was sectioned as if it were a piece of tissue. The embroyos of some of the eggs were removed aseptically a short time after intravenous inoculation to provide explants for transplantation to the chorio-allantois of other eggs or for tissue culture.

Harvesting.—One week after inoculation, on the 18th day of incubation, the eggs were opened into a wide-mouthed container that contained 10 percent formalin. (Under ideal circumstances the chorio-allantoic membrane is not torn during harvesting, and the entire contents of the egg rests in the 10 percent formalin as a single mass.) In 10 or 15 minutes the specimen was transferred to a dish. The chorio-allantoic membrane was incised and removed from the remaining parts of the egg. The yolk sac was removed from the embryo, opened partially, and the fluid contents gently rinsed away. The embryo was opened and dissected so that the various tissues in the abdominal and thoracic cavities received adequate fixation in the 10 percent formalin.

Chorio-allantoic membrane transplants.—The technique used for the transplantation of tissues from the inoculated embryo onto the chorio-allantoic membrane of other eggs is one in general use (*11*). The recipient eggs were harvested on the 18th day of incubation.

Organ fragments in sponge-matrix tissue culture.—Organs were removed from the eggs that had just been inoculated intravenously. Explants of these organs were placed on the surface of a cellulose sponge matrix. Two or 3 explants were used; they were arranged so that they did not touch each other and were cemented in place with a chick plasma clot. One or more ml of feeding mixture was given to the cultures and replenished from time to time as indicated by the drop in *p*H. The feeding mixture consisted of 40 percent human serum in Hanks' balanced salt solution supplemented with the components of Eagle's basal medium.

EXPERIMENTS

Walker Carcinosarcoma 256 in Ascites Form

The first of the embryos to receive the Walker tumor intravenously showed on microscopic examination that the mesonephros was much more extensively involved by tumor growth than the metanephros. In view of the earlier observations of Wolff (*3*), we found this exciting. We repeated our experiment in several series. In multiple sections through the mesonephros and metanephros of more than 40 embryos that received Walker tumor cells intravenously, in every case where tumor cells were

TABLE 1.—Data on transplantation and culture of organ fragments from chick embryos bearing intravascular emboli of ascites Walker tumor cells

12/28/61	Sixty-one 11-day embryos inoculated intravenously with Walker ascites.	
	Forty-one remained for incubation.	Twenty opened promptly; mesonephri removed and cut into explants. Pool of explants from each donor divided into 3 parts: 1 part fixed in 10 percent formalin; 2 parts, each inoculated on chorio-allantoic membrane of 9-day embryo (total of 40 inoculated).
1/4/62	Forty-one eggs opened; 22 alive. Eighteen live embryos fixed with routine described in text (16/18 positive). Four live embryos opened aseptically. Mesonephri of each identified, cut into explants, and divided into 4 parts: 1 part fixed in 10 percent formalin; 1 part in sponge-matrix tissue culture; 2 parts, 1 in each of 2 9-day embryos on chorio-allantoic membrane.	Thirty-six eggs alive. Transplants accepted in 15.
1/13/62	Chorio-allantoic membrane transplants harvested. Six of 8 eggs alive (6/6 positive). Four tissue cultures fixed.	

found in parenchymal organs (above 90%), the degree of mesonephric involvement far exceeded the degree of growth seen in the metanephros. As a result, the invaded mesonephros was the first tissue studied by transplantation to the chorio-allantois and in tissue culture (table 1).

Sixty-one embryos, all 11-day, were inoculated with 0.025 ml suspension of Walker cells from a rat bearing the tumor in ascites form. Twenty of the 61 inoculated were opened a few minutes after inoculation. The mesonephros from the 20 donors was removed aseptically, cut into fragments 1 to 2 mm in diameter, and a few of the fragments from each embryo were promptly fixed in 10 percent formalin for histologic examination. The remainder of the fragments for each of the 20 donors was divided in 2 parts and placed on the chorio-allantois of two 9-day embryonated eggs, *i.e.*, 2 new eggs were used for each of the original 20 donor embryos.

The 41 remaining eggs that had received the intravenous inoculation were placed in the incubator. One week later, 22 of these 41 were still alive. Eighteen of the 22 embryos were harvested and each placed in a large volume of formalin as described previously. The last 4 of the 22 were harvested with aseptic technique, the mesonephri of each of these removed and cut into explants. The explants were distributed as follows for each pair of mesonephri; some on the chorio-allantois of two 9-day recipients, some in sponge-matrix tissue culture, and the remaining fragments in 10 percent formalin.

Of the 18 eggs that had been fixed in formalin and examined with multiple microscopic sections, 16 were extensively involved with tumor implants in many tissues of the embryo and in the extraembryonic membranes. The extensive dissemination involved the chorio-allantois, voluntary muscle, skin, spinal cord, brain, liver, lung, and mesonephros (figs. 1 through 4). The observation which had been made consistently in several series of inoculations that mesonephros is much more extensively involved than metanephros was reconfirmed. Representative illustrations of the appearance of metanephros and mesonephros are seen in figures 5, 6, and 7. The accompanying legends provide descriptions of the microscopic appearance of these growths.

Returning to the 40 transplants of mesonephros on the chorio-allantoic membrane (from the original 20 donors), 36 were alive and in 15 of these the transplanted tissue was accepted by the membrane. In all these transplants a nodule was produced in which Walker tumor cells and mesonephric tubules could be identified. The growth of the Walker tumor in this situation exceeded the growth observed in mesonephric tissue that was left undisturbed *in situ* after intravenous inoculation for the same period (*compare* figs. 7 and 8).

Finally to be considered in this experiment is the appearance 9 days later of the mesonephric fragments from four 18-day embryos that had been inoculated with tumor intravenously 1 week earlier and transferred to the chorio-allantois of 9-day embryos and to sponge-matrix tissue culture. Figure 7 is representative of what was seen in the mesonephric fragments inoculated in this small series. In all 6 embryos that were alive 9 days after inoculation, the transplants were accepted by the chorio-allantois. On microscopic examination growth consisted almost entirely of tumor. Only occasional atrophic tubules of mesonephric tissue could be seen (fig. 9). The 4 sponge-matrix tissue cultures were also incubated for 9 days. The microscopic picture varied, and this small series is difficult to interpret. Growth was not extensive, certainly not comparable to the new growth observed on the chorio-allantois. In some explants there appeared to be fewer Walker tumor cells in the fragments of mesonephros after 9 days *in vitro* than were found in the donor fragments from the original inoculum (fig. 10). In other explants viable mesonephric tissue was not seen, and the explant consisted of Walker tumor cells in a matrix of necrotic remnants of the mesonephros (fig. 11).

The data presented on the fate of transplants of mesonephros bearing Walker tumor cells to the chorio-allantois and to tissue culture are fragmentary. Since only one experiment is described, nothing can be said about its reproducibility. The experiment does illustrate, however, that in transplants of this organ the fate of blood-borne inocula can be followed on the chorio-allantois of other eggs and in tissue culture. Histologic examination permits a comparison of the growth and spatial arrangement of tumor cells and mesonephric tubules. The observations suggest that the 11-day mesonephros, seeded by way of the blood stream with Walker tumor cells and transplanted to the chorio-allantois of 9-day embryos, is the site of better growth for tumors than is the mesonephros left *in situ* in a host bearing tumor-cell emboli in many organs. Future plans will involve a comparison of transplants of mesonephri bearing tumor cells to the chorio-allantois of normal hosts and of hosts modified by the prior intravenous inoculation of tumor cells.

Yoshida Ascites Hepatoma 7974

The results obtained on inoculation of a fresh suspension of these ascites tumor cells in the 11-day embryo were of interest both in the organs of the embryo and in the extraembryonic membranes (fig. 12). The appearance of the tumor nodules on the chorio-allantoic membrane is seen in figure 13. The appearance of some involved organs from 18-day embryos is illustrated in figures 14 and 15.

One experiment designed to examine the practicability of inoculation of tumor cells by way of the blood stream into various organs and then to observe the growth of these implants in organs transplanted to the chorio-allantois was conducted as follows (*see* table 2): A small volume, 0.025 ml, of fresh ascitic fluid was inoculated intravenously into each of two 11-day embryos. The embryos were promptly opened and the following tissues isolated: chorio-allantoic membrane, liver, heart, and intestine. The organs from each donor were kept separate, cut into explant size (about 2 mm in diameter), and for each organ mentioned, 8 chorio-allantoic membrane transplants were prepared first for 1 donor and then the other, *i.e.*, 16 recipients for each organ. The transplants were removed and fixed in 10 percent formalin 1 week after inoculation. Examples of the results of the harvesting are illustrated in figures 16, 17, and 18. Multiple small nodules of tumor growth were seen in each of the organs. The legends accompanying the figures describe the microscopic picture.

This experiment indicated the feasibility of a comparison of the fate of tumor emboli in an undisturbed organ and in organ fragments transplanted to the chorio-allantois of other embryos.

In a subsequent experiment, an effort was made to compare the fate of hepatoma emboli in the heart transplanted to the chorio-allantois with their fate in fragments of heart cultivated in sponge-matrix tissue culture. The period of maintenance on the chorio-allantois and in sponge matrix was 9 days. Unfortunately, on histologic examination no comparison between the fate of the tumor cells in these 2 sites could be made, since the transplants to the chorio-allantois from this single donor were all sites

TABLE 2.—Data on transplantation of organ fragments from chick embryos I and II bearing intravascular emboli of hepatoma ascites tumor cells

		Number inoculated	Number survived	Number of takes of transplants	Number of tumors identified
Intestine	I	8	6	4	3
	II	8	6	3	3
Liver	I	8	6	6	4
	II	8	6	2	2
Heart	I	8	4	3	3
	II	8	5	3	3
Chorio-allantoic membrane	I	8	4	2	2
	II	8	6	5	5

of inflammatory reaction and poor survival of heart tissue was observed. Survival of tumor cells was seen in only one instance. In the tissue cultures the fragments of heart fared better. A careful search through serial sections was rewarded by the identification of rare hepatoma cells in most of the fragments of chick embryonic heart (fig. 19). Almost all of the cells thus identified were in endothelial-lined channels. A different picture has been observed in a small number of organ cultures inoculated with human cell lines.

Human Cell Lines KB and HeLa

We prepared suspensions of cell lines by scraping the walls of culture bottles. The cells were concentrated by centrifugation, and 0.025 to 0.05 ml was inoculated intravenously in a chorio-allantoic vein.

A suspension of KB cells was inoculated into an 11-day chick embryo. The embryo was immediately removed and the heart cut into explants averaging 1.5 mm in diameter. Six sponge-matrix cultures were prepared, each with 2 explants. Two cultures were fixed in 1 week and embedded on edge for histologic section. KB cells were identified in each of the 4 explants examined and were consistently found within the interstitial tissue, often in close contact with myocardial cells, and not in endothelial-lined channels (figs. 20 and 21).

A suspension of HeLa cells was inoculated into three 16-day embryos. The hearts were removed, pooled, and cut into explants. Ten sponge-matrix cultures were prepared. The cultures were all fixed 9 days later. On histologic section, HeLa cells were identified in all the cultures. With rare exception, the HeLa cells were located in the interstitial connective tissue of the heart, often in intimate contact with heart muscle cells (figs. 22 and 23).

DISCUSSION

The development of the methods described in this paper grew out of a need for more adequate systems for a comparison of the interaction between tumor and normal cells under a variety of circumstances, from the situation occurring in the intact host through a series of intermediates—*e.g.*, transplantation of organs, culture of organ fragments—to directly visible microscopic interactions in living preparations.

The ill-defined entity of cancer has as its essential identifying feature a functional property, its ultimate capacity for invading and destroying normal tissue. Of the many items that may be tabulated under the general heading of the physiopathology of cancer, the function least well understood is this critical one, the destructive invasion of normal tissue. The difficulty of the problem is appreciated when one compares the variety of intercellular patterns seen on microscopic examination of clinical cancer with the limited body of knowledge on the mechanisms of tumor spread obtained from experimental systems. The author believes that significant progress in this area requires integration of what is now a confusing tangle of observations of intercellular action. The beginnings of an orderly description exist in the observation of such phenomena as contact guidance, contact inhibition, aggregate replication, and the interplay between parenchymal and stromal cells.

The mechanisms by which intravascular tumor cells produce metastases may be more varied than is usually supposed. Since 1-week-old metastases of cells from 2 different rat ascites tumors are so different in their structure, the earlier stages in the production of these nodules may also differ, perhaps even the initial events of interaction at the site of contact between tumor cells and endothelial surface. This topic is now under investigation.

In this connection the varying ability of normal tissues to act as soils for growth of metastases is a specific topic requiring much more study. Wolff's observation that a variety of tumor cells in tissue culture thrive on explants of mesonephros and our observation that Walker tumor cells administered intravenously in the embryonated egg grow well in mesonephros but not in metanephros may be related to one another. First, however, we must rule out the possibility that the difference observed in our system at the end of 1 week of incubation does not reflect primarily a difference in size of the initial inoculum reaching these 2 organs.

The studies presented here demonstrate the technical feasibility of examinination of the fate of tumor-cell emboli in organ-fragment culture. Preliminary observations suggest that results may vary with at least one selected variable, the choice of the suspension of tumor cells inoculated.

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PLATES

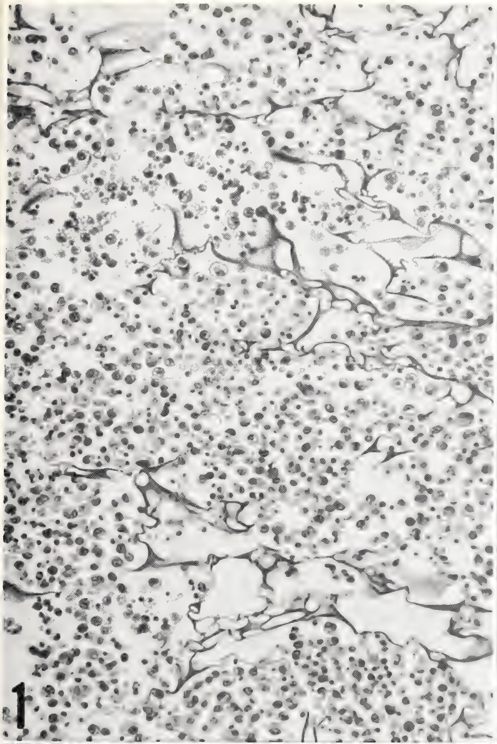
Plate 19

Figure 1.—Walker carcinosarcoma ascitic fluid sopped up in a piece of Gelfoam sponge and then treated with routine histologic technique. Many of the cells are vacuolated. Population is varied, consisting of tumor cells, leukocytes, and red blood cells. Tumor cells show no tendency to form groups. Hematoxylin and eosin. × 135

Figure 2.—Chorio-allantoic membrane of 18-day embryo prepared as a total mount. On 11th day of incubation 0.025 ml of Walker tumor ascitic fluid was inoculated in vein of chorio-allantois. This field shows 2 of the myriads of small, poorly circumscribed tumors that were seen in the membrane after 1 week of incubation. Hematoxylin. × 135

Figure 3.—Microscopic section of lung of 18-day embryo that had received an intravenous inoculation of Walker ascites cells on 11th day of incubation. Many areas of tumor growth are seen between the lobules of the lung. Hematoxylin and eosin. × 135

Figure 4.—Microscopic section of liver of same embryo seen in figure 3. Liver is diffusely infiltrated by Walker tumor cells but the stromal response seen in the lung (fig. 3) is not found here. Hematoxylin and eosin. × 135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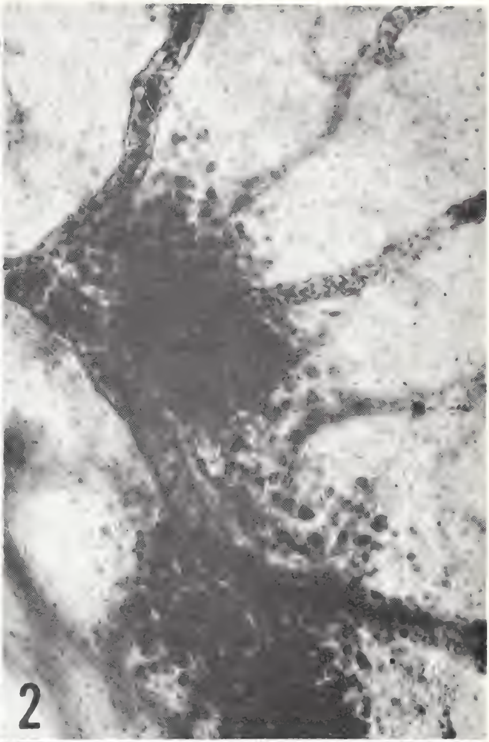
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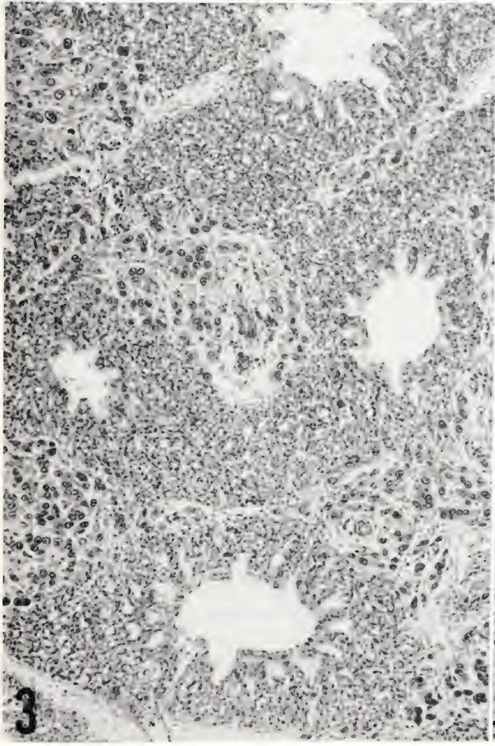
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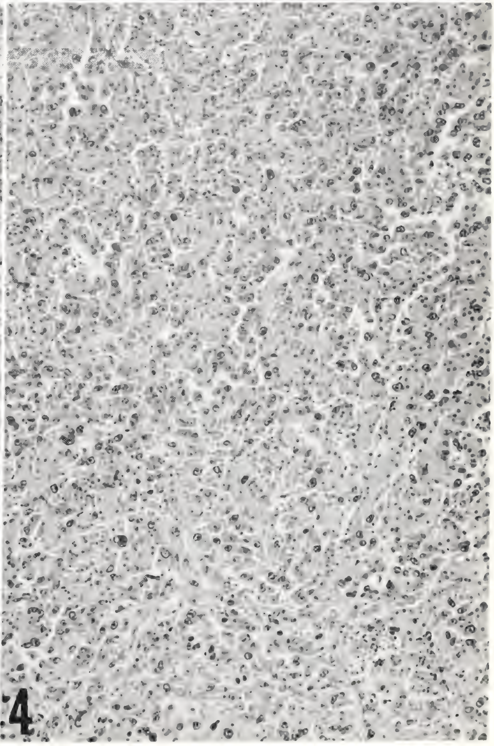
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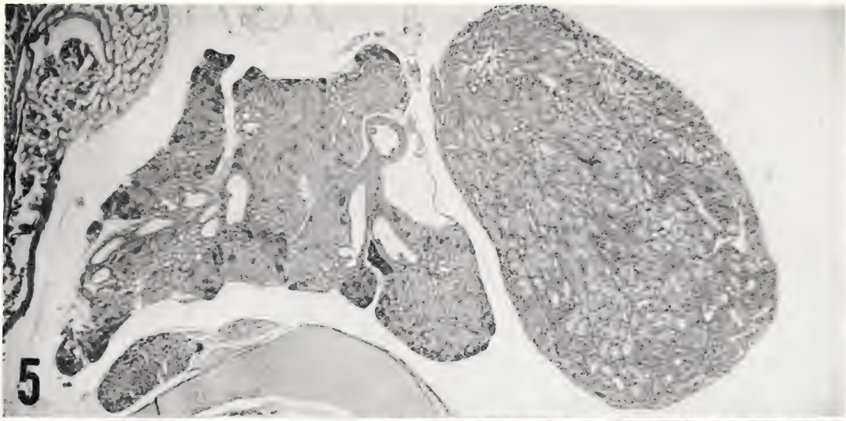
LEIGHTON

PLATE 20

FIGURE 5.—Mesonephros (*right*) and metanephros of 18-day embryo seen with low magnification. On 11th day of incubation the embryo had received 0.025 ml of Walker tumor cells intravenously. The mesonephros is diffusely infiltrated by Walker tumor cells. Hematoxylin and eosin. × 20

FIGURE 6.—Metanephros of figure 5, seen with higher magnification. Occasional Walker tumor cells are seen. A few are found in *upper left corner* of figure near a large tubule; others are seen singly here and there. Three tumor cells are found in prominent vascular channel to *right of center* of photograph. Hematoxylin and eosin. × 135

FIGURE 7.—Mesonephros of figure 5, seen with higher magnification. There is massive diffuse infiltration of the stroma of the mesonephros by Walker tumor cells. Hematoxylin and eosin. × 135



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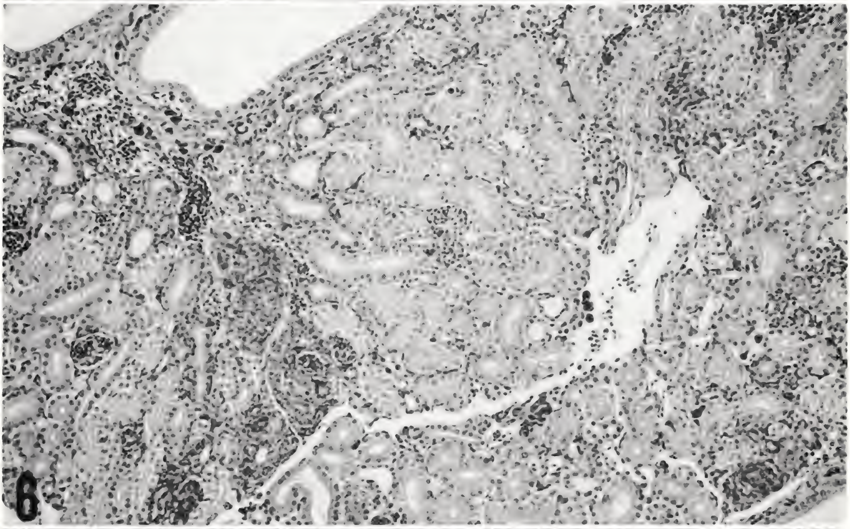

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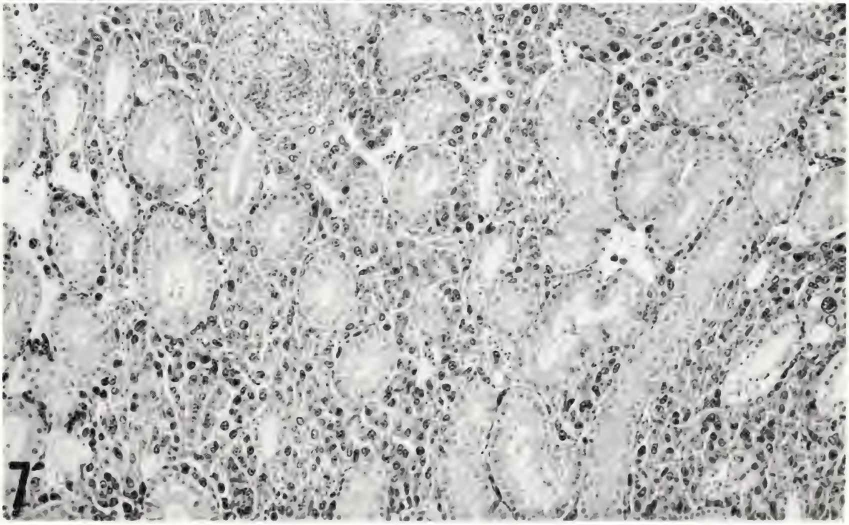

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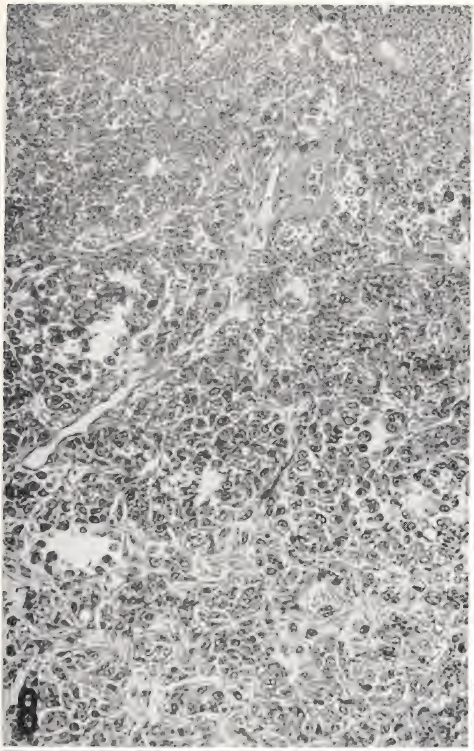
PLATE 21

FIGURE 8.—Histologic section of transplant of mesonephros from 11-day embryo to chorio-allantoic membrane of 9-day recipient. Transplant was harvested 1 week after inoculation. Donor egg had been modified by intravenous inoculation of 0.025 ml of Walker tumor ascitic fluid a few minutes before the egg was opened. Although histologic sections of donor fragments contained only occasional Walker tumor cells in the vascular sinusoids, the transplants as seen here 1 week later consist for the most part of massive growth of Walker tumor cells with some mesonephric structures surviving. One such residual tubular structure is seen in *upper part* of figure. Tumor cells are seen in the lumen of tubule. The degree of tumor growth exceeds that found in mesonephric tissue of animals that were not disturbed after inoculation of tumor cells. Hematoxylin and eosin. × 135

FIGURE 9.—Histologic section of transplant of mesonephros from 18-day donor, inoculated intravenously 1 week earlier, on the chorio-allantois of a 9-day recipient. Recipients were returned to incubator for 9 days until their 18th day of incubation. At this time the transplants consisted almost entirely of tumor in which occasional remnants of mesonephros were found with great difficulty. One cystic thin-walled structure seen in the *upper part* of figure probably represents a mesonephric residuum. Hematoxylin and eosin. × 135

FIGURE 10.—Fragment of mesonephros similar to the one transplanted to egg in figure 9 which was placed in organ culture on sponge matrix and fixed 9 days after preparation. Surface of the explant appears viable, and there has been a rearrangement of epithelial mesonephric cells forming a covering for the explant. Some Walker tumor cells are seen in the interstitium of the transplant. The tumor has fared badly as compared with figure 9. Hematoxylin and eosin. × 135

FIGURE 11.—Fragment of organ culture similar to the one in figure 10 in its preparation and maintenance. Mesonephric tissue is necrotic, but Walker tumor cells appearing viable are numerous. Hematoxylin and eosin. × 135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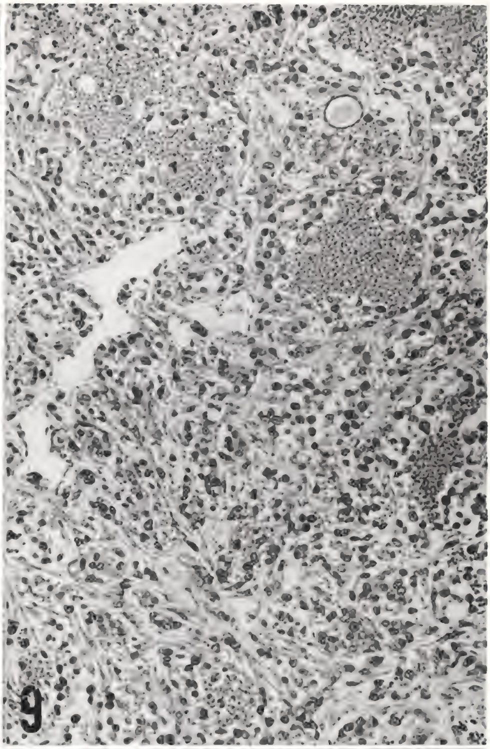
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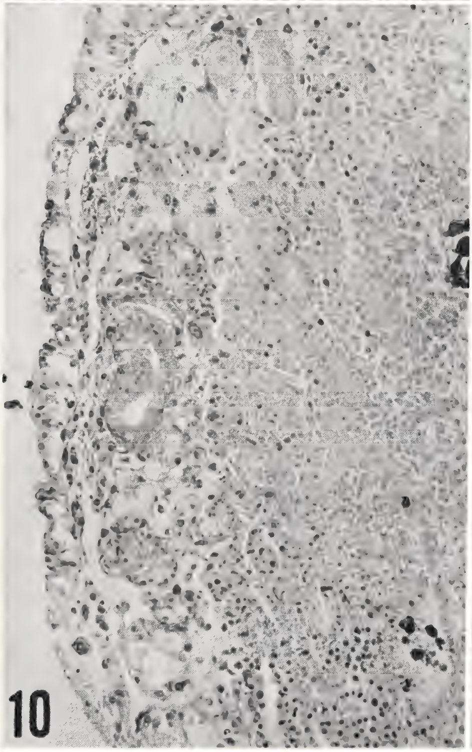
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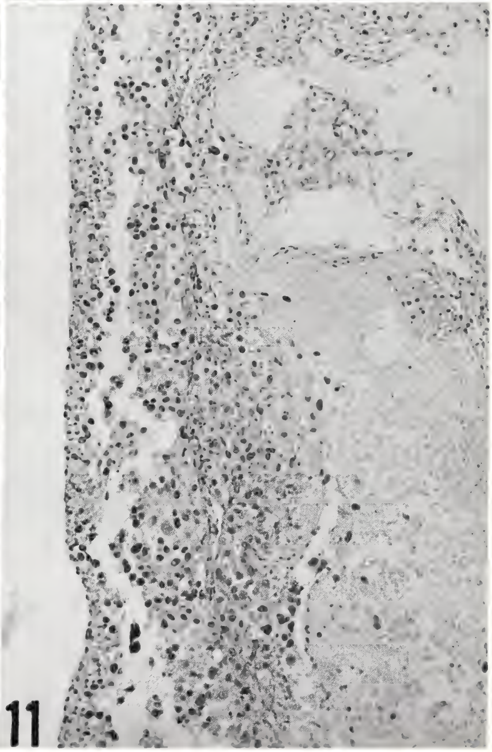
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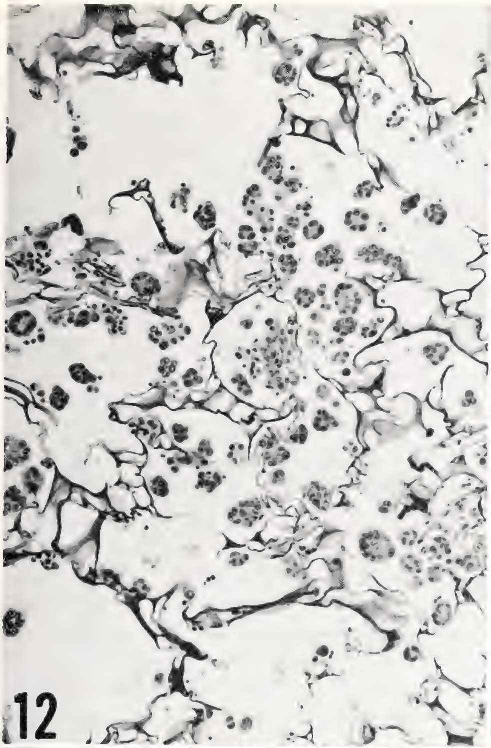
Plate 22

Figure 12.—Microscopic section through a suspension of Yoshida ascites hepatoma 7974. Ascitic fluid was sopped up in a piece of Gelfoam sponge and the sponge then treated with routine histologic technique. In this field most cells are in groups of varying size. There are occasional single cells. The frequency of single cells and groups of cells varies from field to field. Hematoxylin and eosin. × 135

Figure 13.—A total mount prepared from the chorio-allantoic membrane of an 18-day embryo that had received an intravenous inoculation of Yoshida ascites hepatoma cells on 11th day of incubation. Chorio-allantoic membrane was stippled with minute tumors. Several typical ones are illustrated in this figure. Tumors are globular with distinct margins and vary considerably in their size. Appearance of these transplants may be compared with that of the transplants of Walker ascites inoculated in the same way as illustrated in figure 2. Hematoxylin. × 135

Figure 14.—Histologic section through lung of 18-day embryo. On 11th day of incubation, embryo was inoculated with Yoshida hepatoma cells intravenously. Many nodules of tumor are seen throughout the lung. Hematoxylin and eosin. × 135

Figure 15.—Histologic section of embryonic liver from 18-day embryo. Hepatoma cells were inoculated intravenously on 11th day of incubation. Tumor growth is extensive in the liver and is recognized as collections of large cells with prominent, large nuclei. Hematoxylin and eosin. × 135



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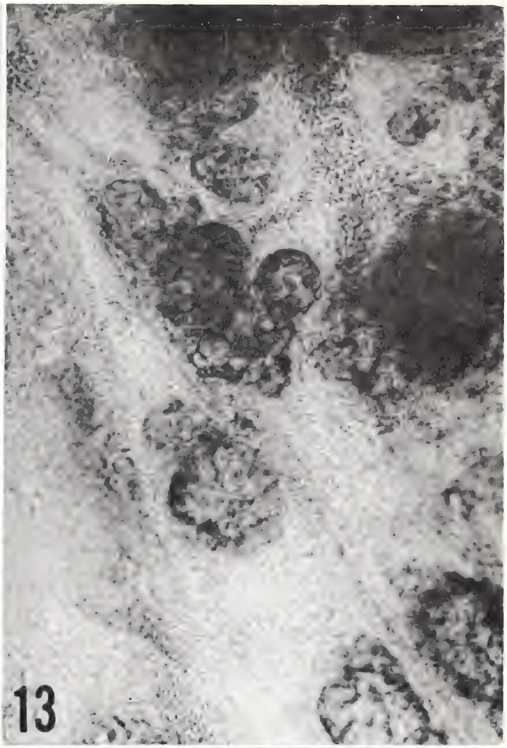

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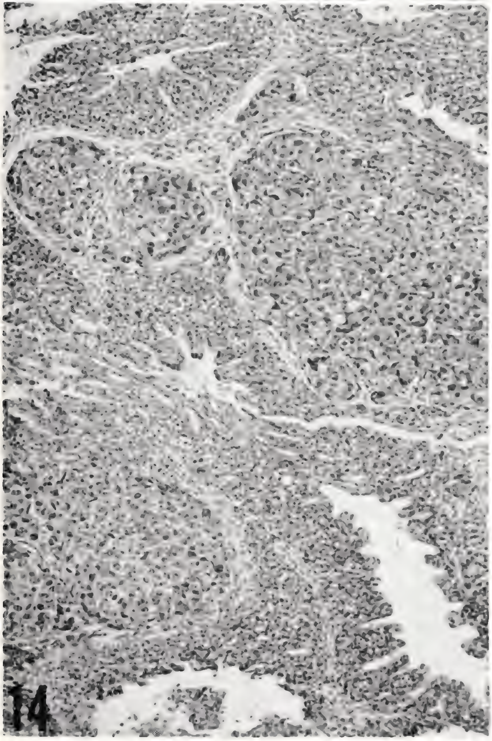

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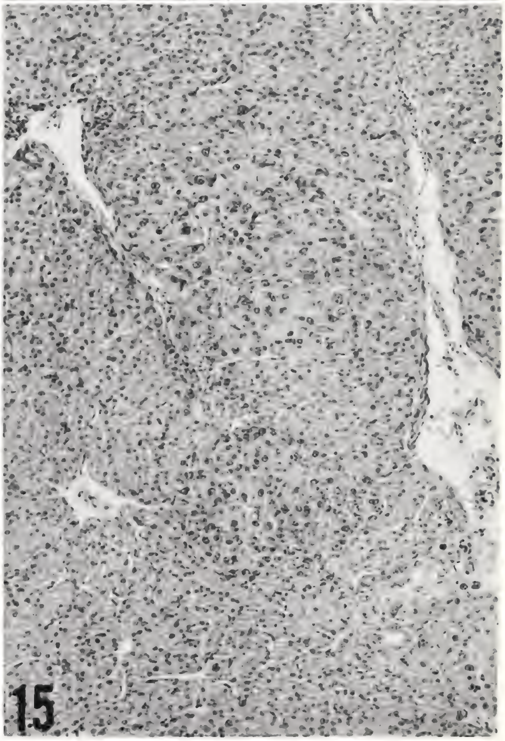

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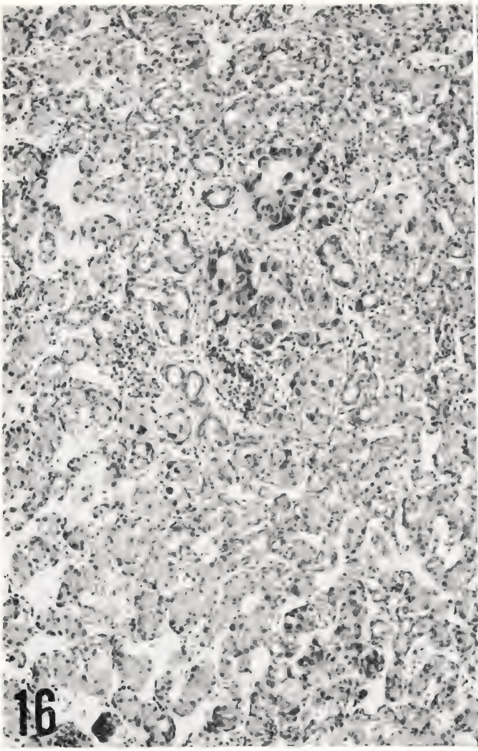
PLATE 23

FIGURE 16.—Microscopic section of transplant of 11-day chick embryo liver on the chorio-allantois of 9-day recipient. Just prior to removal of the embryonic liver, the 11-day donor received an intravenous inoculation of Yoshida ascites hepatoma cells. On examination of fragments of the liver from the donor, rare tumor cells were found in vascular sinusoids (not illustrated here). One week after transplantation there are numerous foci of tumor growth in the transplanted liver. In some instances, as seen in this figure, tumors are in portal areas and are accompanied by proliferation of bile ducts. Hematoxylin and eosin. × 135

FIGURE 17.—Microscopic section through fragment of chick embryonic intestine from 11-day donor prepared as in figure 16 and transplanted to 9-day recipient. One week later tumor growth is seen commonly in the subserosal connective tissue (*upper part* of figure) and is occasionally found in the stroma of villus processes (*lower part* of figure). Hematoxylin and eosin. × 135

FIGURE 18.—Transplant of chick embryonic heart from donor prepared as in figures 16 and 17. There are multiple areas of tumor growth in transplant. Hematoxylin and eosin. × 135

FIGURE 19.—Microscopic section through organ-fragment culture in sponge matrix of chick embryonic heart from an 11-day donor prepared in the same way as the donors of figures 16, 17, and 18. Culture was maintained *in vitro* for 9 days. The focus in which the greatest number of hepatoma cells was found is illustrated here. Tumor cells are within a vascular channel lined by endothelial cells. The tumor cells have not entered interstitial tissue of the heart. In *lower part* of figure a tumor cell appears at the orifice of a narrow endothelial channel and not in the interstitial tissue. In other areas in this series, smaller numbers of hepatoma cells were seen. Almost all tumor cells were found within the lumens of endothelial-lined channels. Hematoxylin and eosin. × 210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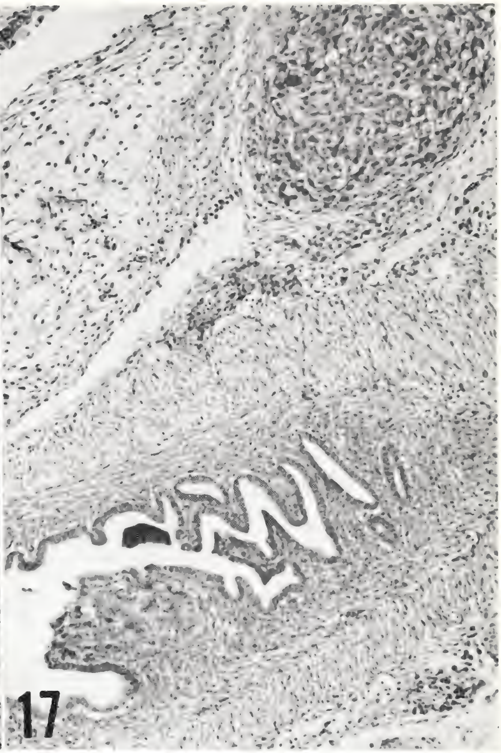
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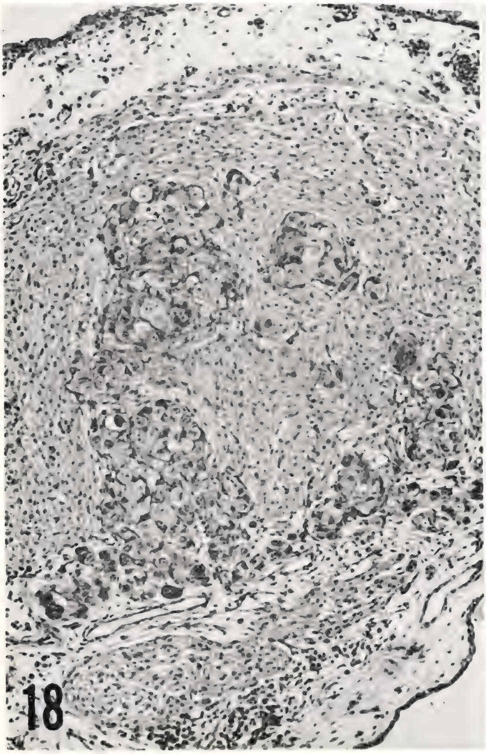
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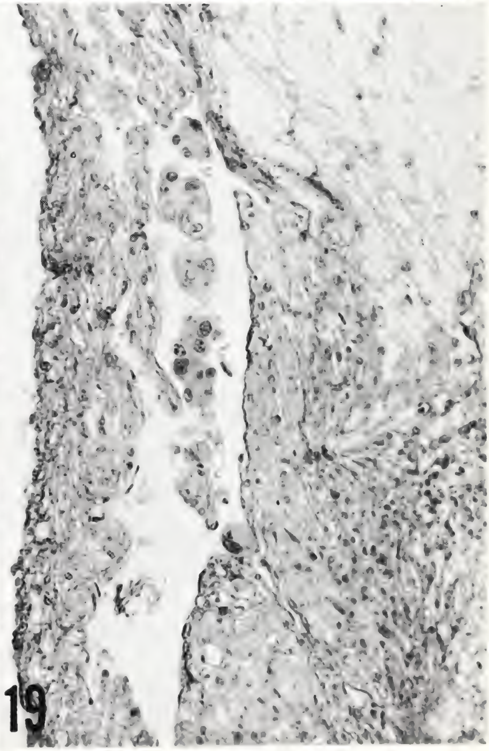
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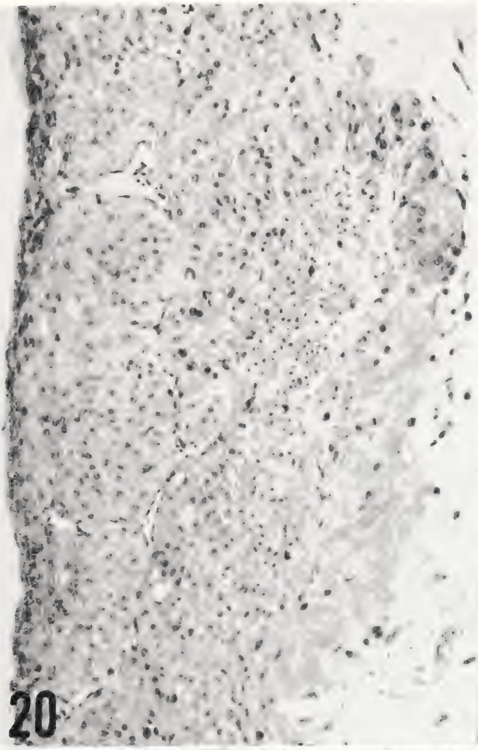
Plate 24

Figures 20 and 21.—Microscopic sections through two 7-day organ-fragment cultures in sponge matrix of chick embryonic heart. The donor was an 11-day chick embryo that had received a suspension of KB cells intravenously just before the cultures were made. *Near the centers* of each figure, KB cells are recognized by their hyperchromatic, large nuclei, and prominent nucleoli. The KB cells are not in endothelial-lined channels, but appear to be in direct contact with myocardial cells. Hematoxylin and eosin. × 210

Figures 22 and 23.—Microscopic sections through two 9-day organ-fragment cultures in sponge matrix of chick embyro heart. Donor fragments were obtained by pooling the hearts of three 16-day embryos and cutting the tissue into pieces of explant size. Donor embryos had received a suspension of HeLa cells intravenously just before cultures were made. The HeLa cells are recognized as large hyperchromatic cells with prominent nuclei and nucleoli. In each of the organ fragments most tumor cells are in intimate contact with myocardial cells. However, in one focus, *near the center* of figure 22, HeLa cells are seen at several sites along the course of a narrow, endothelial-lined channel. Hematoxylin and eosin. × 210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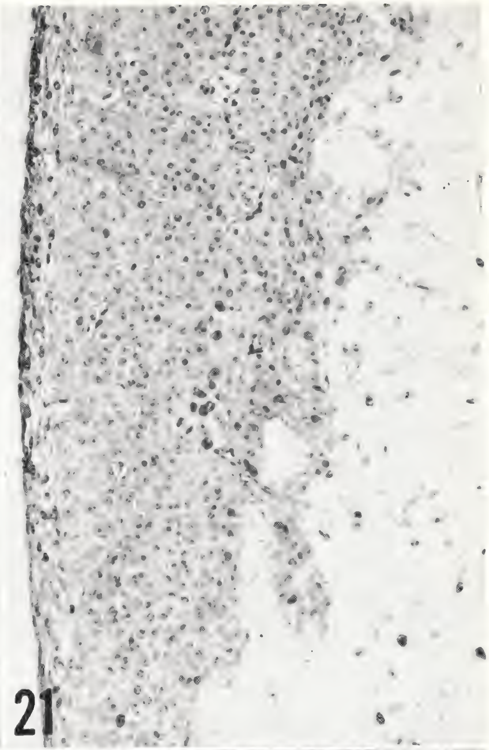
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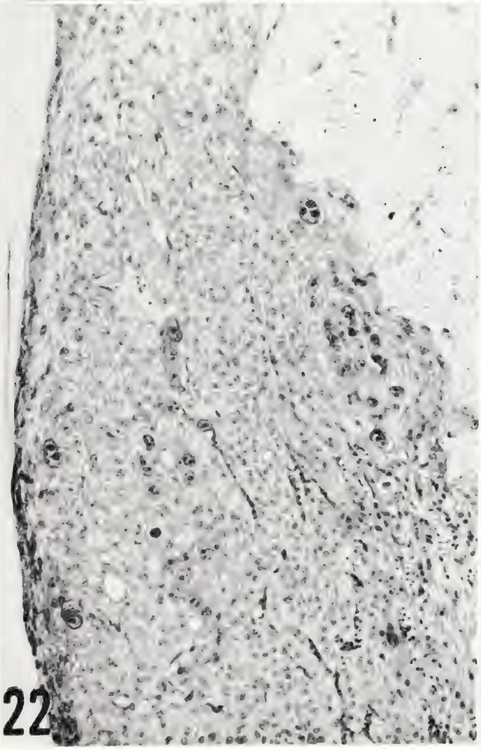
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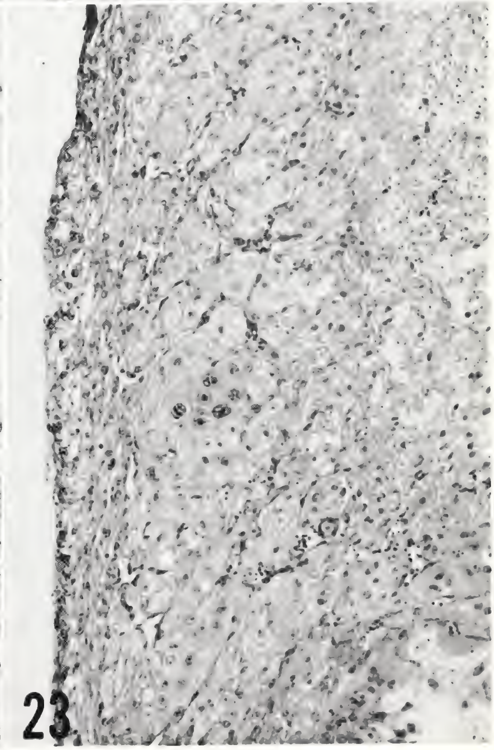
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DISCUSSION

Long-Term Organotypic Cultures of Human Surgical Tumors at the Expense of Substances Elaborated by the Mesonephros of the Chick Embryo. ETIENNE WOLFF, Laboratoire d'Embryologie Expérimentale du Collège de France et du Centre National de la Recherche Scientifique, Paris, France

Dr. Leighton's report gives a remarkable confirmation of experimental results initiated several years ago by my collaborators and myself concerning the culture of tumors on explants of mesonephros of the chick embryo (*1–10*). The data presented by Dr. Leighton demonstrate that tumor cells injected in the chick embryo by way of the blood stream proliferate more intensively in the mesonephros than in the metanephros.

This proliferation may have 3 possible explanations: 1) Tumor-cell emboli are especially attracted by the mesonephros; 2) tumor-cell emboli penetrate the mesonephros more easily than other organs; and 3) the mesonephros provides a favorable nutritive environment for growth and proliferation of malignant cells.

Our results of cultivation of tumors with embryonic organs have shed light on this problem. The investigations discussed here are somewhat the counterpart of those presented by Dr. Leighton. They concern the invasion of organs by tumor fragments, which were associated with the organs *in vitro*, isolated from the complexity of the whole organism.

It is certainly of great interest to elucidate the conditions by which cancer cells penetrate into an organ by way of the circulatory system. It is also interesting to know how these cells invade an organ in the absence of blood circulation, in the conditions of an organ-culture system. Observations of cancer cells and embryonic organs *in vitro* make it possible to study: 1) the comparative behavior of tumor cells and normal tissues in direct association; 2) the pathways by which neoplastic cells invade the tissues; and 3) the influence of nutritional factors provided by various embryonic organs on the behavior of tumor cells.

PREVIOUS INVESTIGATIONS

Briefly, the main phases of our investigations of tumors associated *in vitro* with embryonic organs are as follows:

First we cultured mouse Sarcoma 180 on various organs of the chick embryo. The mesonephros was shown to be the most favorable organ for tumor-cell proliferation [Wolff and Schneider, 1957 (*4*), and Schneider, 1958 (*5*)]. The medium utilized for the culture of mesonephros consists of an agar-Tyrode solution, diluted embryonic extract, and horse serum [Wolff and Wolff, 1961 (*11*)]. This medium alone does not permit survival or growth of the tumor. In contact with the mesonephros (or some other organ of the chick embryo), cancer cells invade the normal tissues which they destroy and replace. They feed as parasites at the expense of the mesonephros. Other mouse and rat tumors, myelosarcoma T58, uterine epithelioma T8, mammary epithelioma G6 of the rat, mammary adenocarcinoma T2633 of the mouse [Wolff and Sigot 1961 (*12*)], and a mouse chondrosarcoma [Wolff and Wolff, 1961 (*11*)] were also cultured successfully with this technique.

Several of these tumors were serially transferred from mesonephros to mesonephros for several months, without losing their proliferative power.

We succeeded in culturing in the same way the following human tumor strains: HeLa, KB, Fogh, J111 [Wolff and Wolff, 1958, 1959 (*7, 13*)], Detroit-106, Detroit-6 and H.Ep. #2 (unpublished data). The cells of these strains, when seeded on some embryonic organs, invaded them and proliferated actively, sometimes forming massive nodules. These strains may be grown on mesonephros in long-term cultures.

We obtained further information by placing a dialyzing membrane (the vitelline membrane of the hen's egg) between mesonephros and tumor cells [Wolff, 1961 (*14*) and Wolff and Wolff, 1961 (*11*)]. In this system, the malignant cells cannot feed directly at the expense of embryonic tissues, like parasitic organisms, but they do feed at the expense of substances secreted by the mesonephros that filter through the membrane. Mouse Sarcoma 180 and human tumor strains HeLa, KB, and Fogh were cultured successfully with this method [Wolff and Wolff, 1961 (*15*)].

The cells of mouse Sarcoma 180 retain *in vitro* the morphology and the properties that they show when maintained as a transplantable tumor, *i.e.*, a spindle or stellate shape, a high migratory rate, and much proliferative power. Cells of human strains HeLa, KB, and Fogh reconstitute solid epithelial nodules (figs. 1 through 6), which contrast with the undifferentiated monolayers that they form in routine culture with liquid media. Thus they recover morphological and physiological characteristics of the initial tumor.

CULTURES OF FRESH SURGICAL HUMAN TUMORS

We have recently succeeded [Wolff and Wolff, 1961 (*9, 10, 16*)] in culturing with the previously mentioned techniques, human tumors taken directly from patients during surgical operations or biopsies. Numerous tumors of various types were cultured on the chick mesonephros, such as fragments of tumors of the uterus, stomach, pancreas, liver, lung, and their metastatic growths, and lymphoid tumors. Cancerous tissues retain in such cultures the structure and properties they showed in the initial tumor. Figures 7 through 10 show histological sections of various tumors cultivated for 5 to 10 days on the chick mesonephros.

We shall consider now a hepatic metastasis of a gastric neoplasm, which we have maintained in organotypic culture for 140 days,[1] and which we call Z200.

Primary Culture

Tumor Z200 was removed surgically on January 10, 1961, from a 34-year-old patient.

The medium on which the mesonephros was explanted had the following constitution [Wolff and Wolff, 1961 (*11*)]: 1 percent agar in Gey's solution, diluted 8-day chick embryo extract, and horse serum in a ratio of 7 : 3 : 3.

A piece of vitelline membrane of a hen's egg was stretched on the medium. Small fragments of mesonephros 0.1 to 0.3 mm long and 0.1 to 0.2 mm wide were placed together on the vitelline membrane. The fragments soon fused and formed an almost continuous plate. The tumor pieces were placed either on this plate or in spaces between pieces of mesonephros, forming with them a kind of mosaic. Then the vitelline membrane was folded back over explants, enclosing them in a kind of pocket.

Certain cultures were fixed and examined histologically after 7 days of explantation. It was found that the tumor fragments grew at the expense of the mesonephros. The cultured tumor appeared similar anatomo-pathologically to the tumor from which it was originated. It consisted of anastomotic cords of mucus-secreting epithelial cells. They often formed the wall of cavities which result from lysis of certain cells. The whole nodule had an alveolar and glandular aspect, which corresponded to the primary structure of the tumor (*17*) (figs. 11, 12, and 13). Mitotic figures were numerous.

Long-Term Serial Cultures Through Repeated Transfers

Of 20 primary cultures, 9 were transferred to new media with fresh pieces of mesonephros. Subcultures were made periodically, every 8th day at the beginning and later every 10th to 14th day, because it seemed better to allow the cultures to develop longer on the same medium.

Of 9 primary cultures, which were subcultured, 3 continued to grow after the second transfer. The surviving ones were divided into 2 or more pieces, and some of them were fixed at each passage. Others were transferred onto new media. The number of healthy subcultures was 20, 140 days after the beginning of the experiment.

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[1] On about Nov. 20, 1962, 320 days.

Explants appeared macroscopically as lumpy and translucent nodules, having the aspect of spheres or lenses. They were in intimate contact with the mesonephros, and a flat rim surrounding the nodules corresponded to the zone of invasion (figs. 14,15, and 16). Their growth was rapid and strong. During a period of 8 to 10 days, they increased from 1.5 to 2 times in diameter. The histological structure of explants remained very similar to that of the first culture. One could still observe epithelial cords organized around alveolae (figs. 17 through 20). In some cases, the cavities were more elongated than others and seemed to be more or less flattened, but the general structure was fundamentally the same. The explants still had a solid aspect and an organotypic structure. Mitotic figures were still numerous.

Cultures of Tumors Separated From Mesonephros by a Membrane

After 6 to 7 successive passages, we attempted to apply the technique of the dialyzing membrane to the tumor Z200 [Wolff and Wolff, 1962 (*17*)]. A vitelline membrane was interposed between pieces of mesonephros and a nodule of Z200, previously cultured directly on the mesonephros.

The nodules grew considerably. They increased in the three dimensions, forming thick discs or lenses, having a translucent or milky aspect (fig. 21). After 8 to 14 days, the diameter of the tumor increased from 2 to 4 times. After 7 to 8 passages effected under these conditions (a total of 79 days), the tumor continued to develop at the same rate. It sometimes covered a large surface of the underlying mesonephric plate.

When the vitelline membrane was removed, explants appeared as masses of a viscous and granular jelly. One could displace them on the same medium or transplant them onto another one, without dissociating them. Histologically, they retained their organized structure with epithelial cords, which were however less solid and compact. Their cavities were narrow, stretched, and anastomosed (fig. 22). However, the general organization of tissues and cells did not appreciably differ from the initial structure of the tumor.

Thus it is possible to culture for prolonged periods *pure human tumors in organotypic conditions* in the presence of soluble substances elaborated by the mesonephros.

CONCLUSIONS

The results reported here provide evidence that human cancers surgically removed may be cultured directly on fragments of mesonephros explanted *in vitro*. The tumors preserve their organotypic or, more precisely, *their carcinotypic structure*. It is possible to maintain the cultures for several months, and perhaps indefinitely, through repeated passages, without alteration of structure or diminution of its proliferation power. This suggests that the human neoplasm Z200 receives all necessary nutritional requirements and that it does not contain in itself special growth-promoting substances, which would become exhausted after several passages.

Direct contact between cancer cells and mesonephric tissues is not necessary for maintenance of the tumor. The tumor is able to utilize those substances secreted by the mesonephros which pass through a dialyzing membrane. We know that, in the absence of mesonephros, the malignant cells are not able to survive or to proliferate on the standard culture medium used in our laboratory. The question is raised whether the substances elaborated by the mesonephros provide the tumor with *all necessary nutrients* or whether there are *indispensable growth-promoting* substances, acting together with other nutrients being supplied by the medium.

Evidence is presented in any case that mesonephros provides an environment extremely favorable to the proliferation of cancer cells, thanks to physical conditions which it offers the invading cancerous cells [Wolff, 1956 (*3*) and Wolff and Schneider, 1957 (*4*)] and to chemical substances which stimulate their proliferation. These considerations lead us to an approach of the solution of the question raised by Dr. Leighton's experiments. Tumor-cell emboli are favored by the intrinsic conditions realized in the mesonephros. It appears that growth-promoting factors in this organ stimulate the multiplication of cancer cells and permit a rapid invasion of the mesonephric tissues.

On the other hand, we have demonstrated that organs other than mesonephros—gonads, metanephros, skin, etc.—are able to support the development *in vitro* of cancerous cells. They are, however, less favorable to their proliferation. Perhaps they contain or elaborate a smaller amount of substances that promote tumor growth.

We may finally ask whether human tumors other than Z200, when freshly removed from the patient, are able to nourish themselves indefinitely at the expense of chick mesonephros and if all human tumors are able to proliferate for long periods under these conditions. Our previous investigations (*9, 10, 16*) have shown that numerous human tumors can be fed, in primary cultures, at the expense of the chick mesonephros (*18*). We have recently subcultured other tumors (a metastatic growth of a tumor of the pancreas, 2 carcinomas of the stomach and of the lung) during 30 to 70 days under these conditions (unpublished data).

It is, however, unlikely that all human tumors would have the same tolerance to such an abnormal environment. Perhaps some tumors would not be adequately nourished by chick embryonic organs and would survive for only a limited period. These tumors may probably retain some growth substances and oligoelements, which may support their proliferation for a while. When they are deprived of such substances, cultures may undergo degeneration. We do not know the behavior of different tumors, but it seems probable that differences occur in their nutritional requirements. We intend to analyze these differences in future investigations.

SUMMARY

Numerous surgically removed human tumors were cultured *in vitro* in association with explants of 8-day chick mesonephros. They retained their original structure and characteristics.

A hepatic metastatic growth of the gastric epithelial tumor, designated Z200, was cultured by this technique. When associated with fragments of mesonephros, tumor cells grew as solid nodules, which consisted of epithelial cords surrounding alveolae. There was much proliferation in the cells of the nodules, whose rate of growth was very rapid.

This tumor has been cultured since January 10, 1962, and is being currently maintained. It was serially transferred every 10th to 14th day on fresh cultures of mesonephros. It is still actively growing after 320 days of culture.

Some tumor explants were separated from the mesonephros after the 7th-passage by a dialyzing membrane. They grew rapidly at the expense of dialyzing substances elaborated by the mesonephros. Such explants were subcultured for 79 days and are still living. They formed rather viscous solid nodules with less compact cords than in direct association with the mesonephros. They retained their organized structure and their initial rate of proliferation.

Evidence is presented that it is possible to culture directly fragments of surgically removed human cancer for long periods with explants of chick mesonephros. Explant of the human tumor Z200 may also be cultivated at the expense of substances elaborated by the embryonic kidney. These substances may pass through a thin dialyzing membrane and nourish organotypic culture of human tumors.

Alternative hypotheses to explain the growth-promoting effects of substances elaborated by the chick mesonephros are discussed.

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PLATES

Plate 25

Figure 1.—Nodule of HeLa cells separated from the mesonephros by a sheet of vitelline membrane. × 128

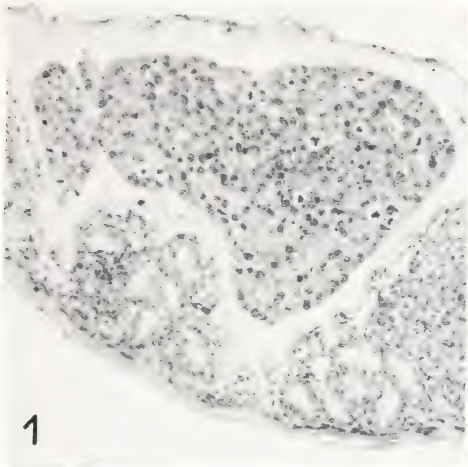
Figure 2.—Detail of nodule of HeLa cultivated *in vitro* under the same conditions, forming a kind of root toward mesonephros, from which it is separated by the vitelline membrane. × 300

Figure 3.—Rim of organotypic culture of HeLa cells, showing several mitotic figures. × 380

Figure 4.—Section of culture of KB cells, separated from mesonephros by piece of vitelline membrane. × 128

Figure 5.—Another culture of KB cells, forming massive nodule with 2 appendages depressing vitelline membrane. × 115

Figure 6.—Detail of appendage as represented in figure 5. × 440



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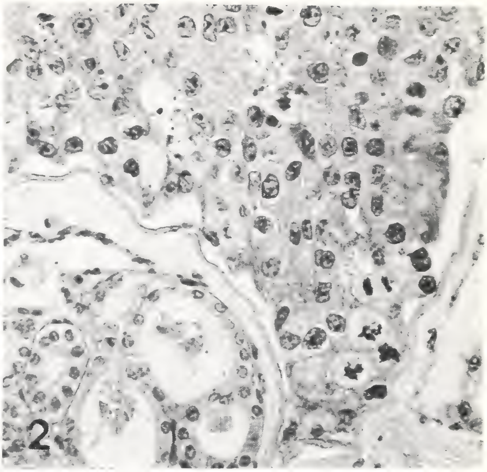

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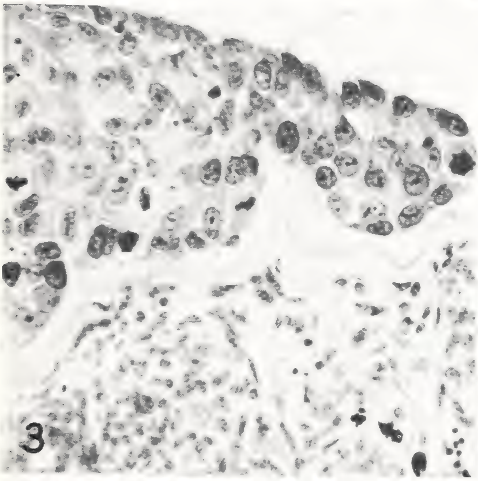

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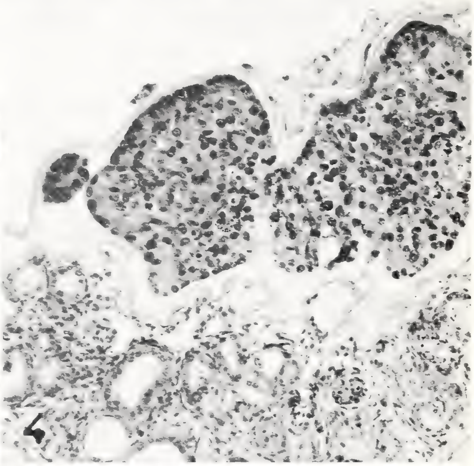

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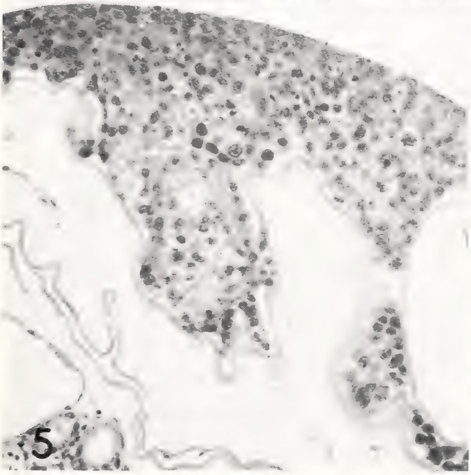

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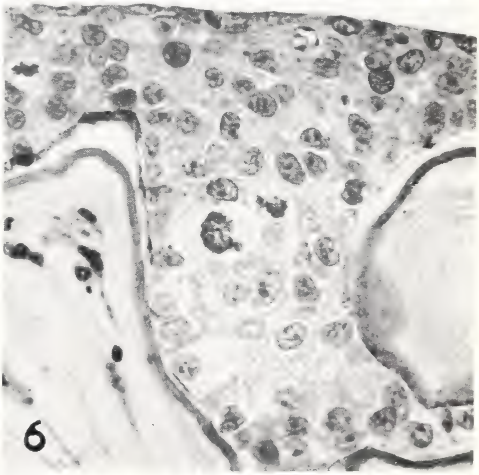

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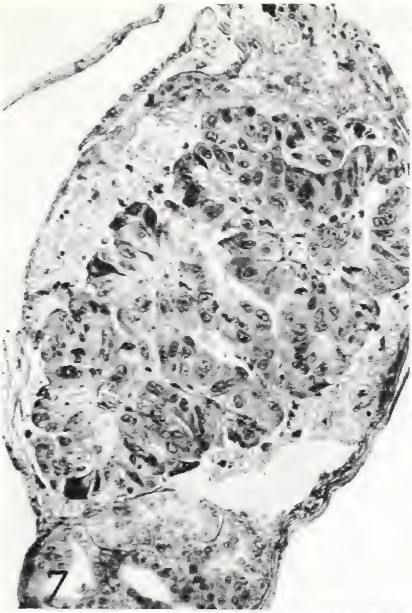
Plate 26

Figure 7.—Section through nodule of human gastric epithelial tumor, directly associated *in vitro* with chick mesonephros. × 175

Figure 8.—Cells of same tumor as shown in figure 7, forming a morula-like nodule in contact with mesonephric cells. × 400

Figure 9.—Another human gastric tumor, developing epithelial cords in association with chick mesonephros. × 355

Figure 10.—Another human gastric tumor, developing epithelial vesicles in contact with mesonephros. × 225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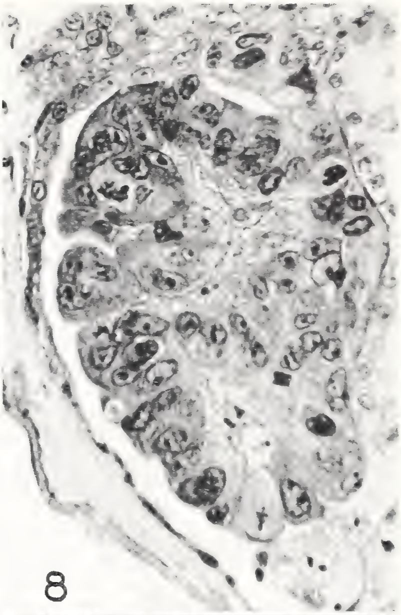
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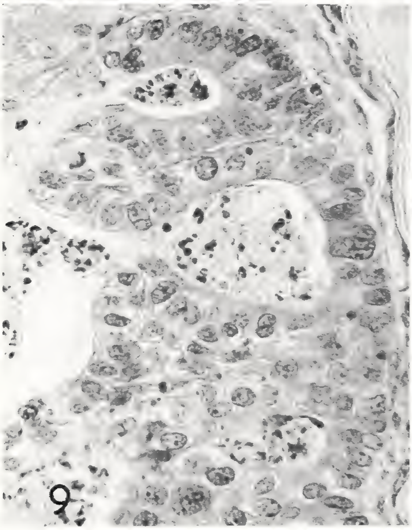
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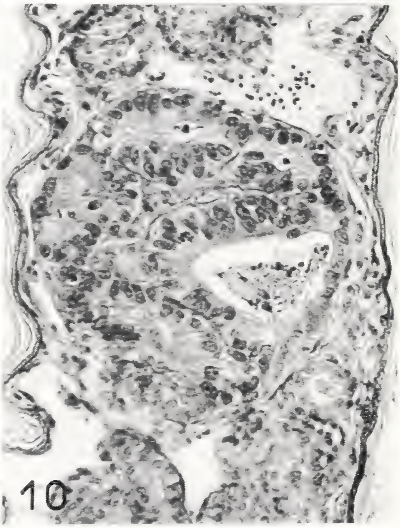
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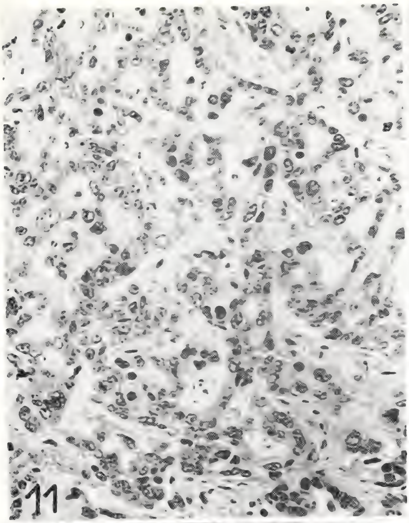
Plate 27

Figure 11.—Hepatic metastatic growth Z200, probably originated from a gastric tumor. Histological section of tumor before culture. × 175

Figure 12.—Primary culture of the same tumor (Z200) after 7 days. *Note* alveolae surrounded by epithelial cells. × 300

Figure 13.—Detail of tumor Z200, after 7 days of culture. × 400

Figures 14, 15, and 16.—General view of living explants of tumor Z200, growing at the expense of mesonephros. Around dark nodules of malignant tissues, mesonephric tubules and glomerulae are distinguishable. × 20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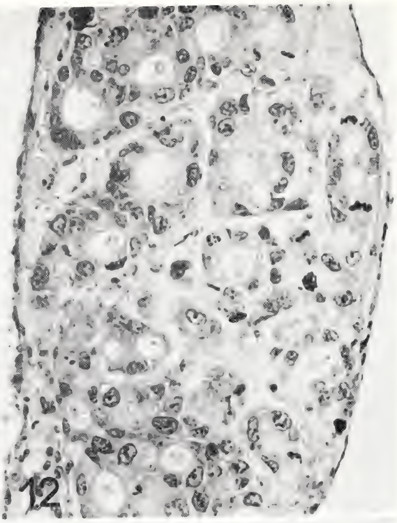
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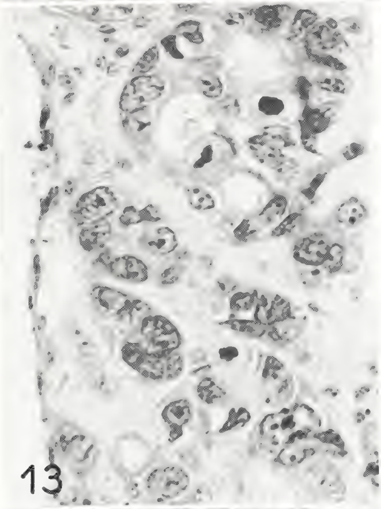
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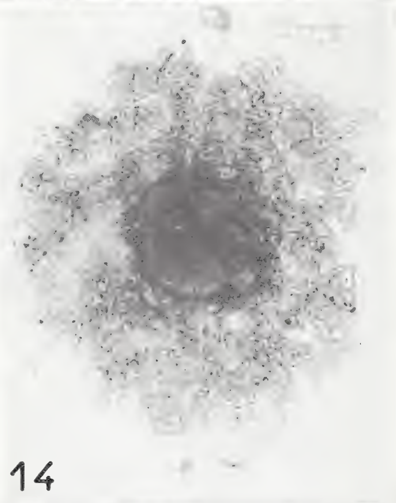
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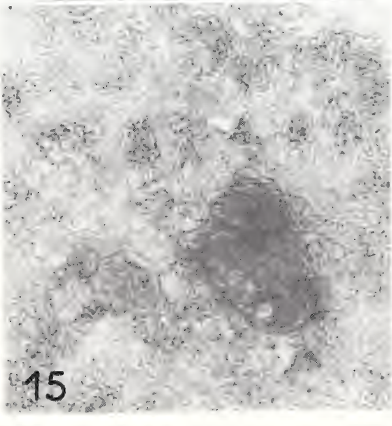
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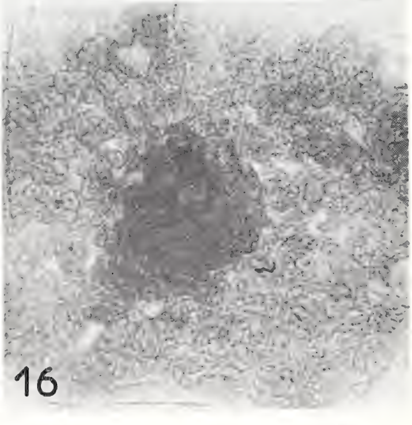
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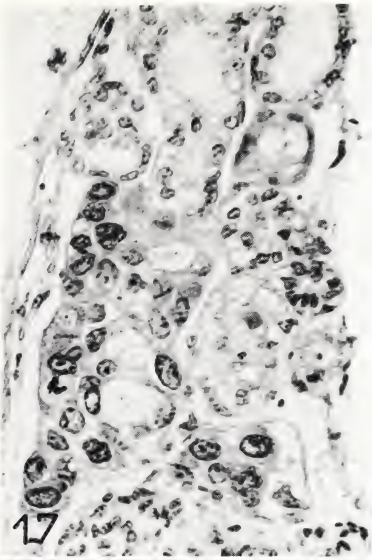
PLATE 28

FIGURE 17.—Epithelial cords of tumor Z200 invading the mesonephros after 3 transfers. × 350

FIGURE 18.—Section of nodule of Z200 in fourth subculture. × 220

FIGURE 19.—Detail of nodule of Z200 in third subculture. × 330

FIGURE 20.—Alveolar structure of tumor Z200 after 8 transfers in association with mesonephros. × 350



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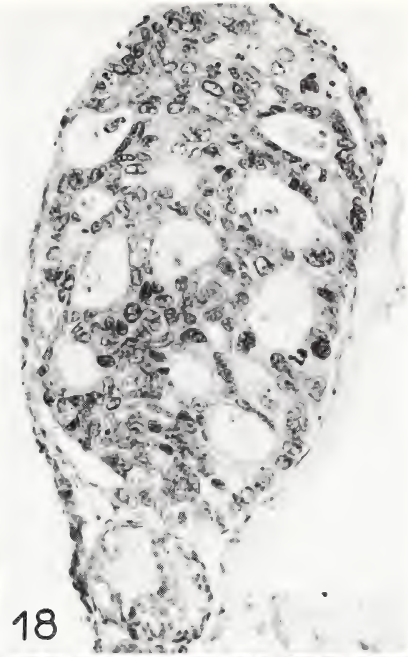

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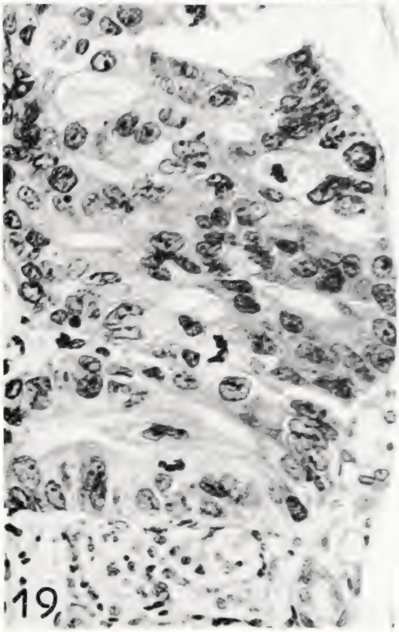

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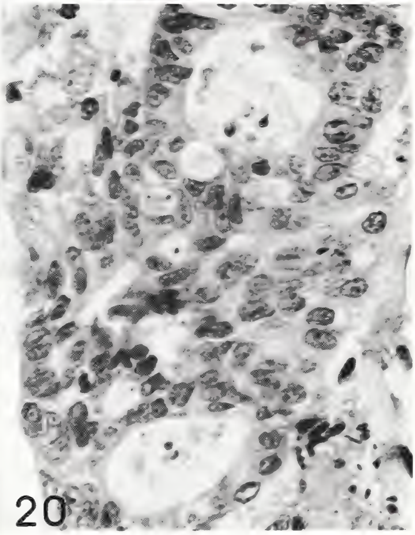

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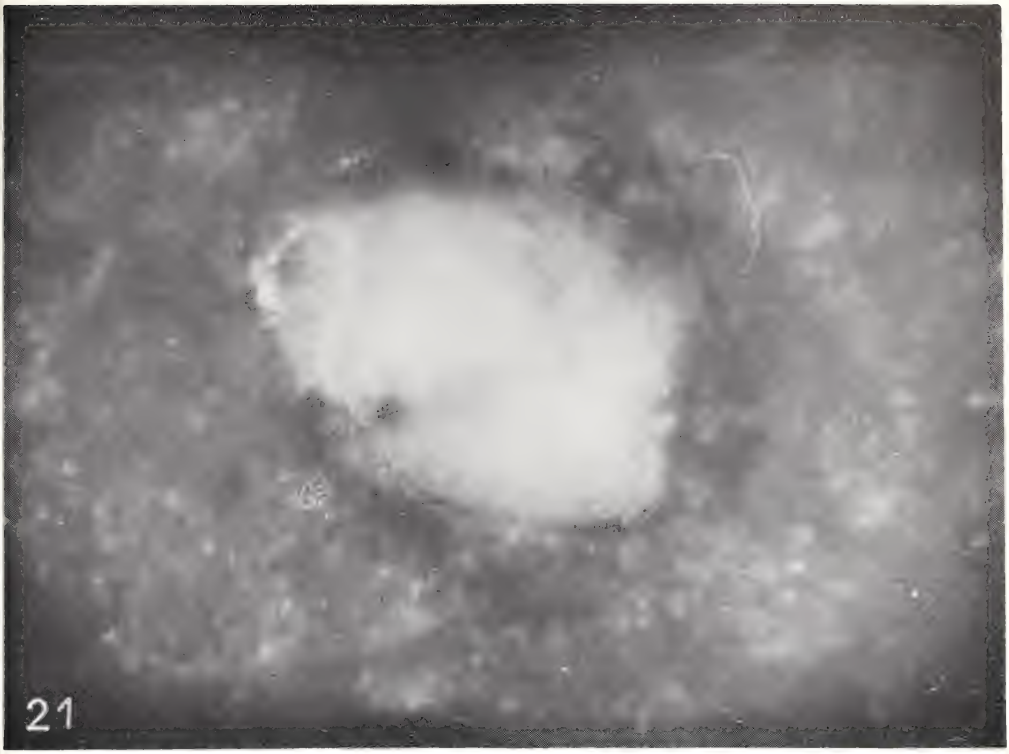
Plate 29

Figure 21.—General view of living nodule of tumor Z200 separated from the mesonephros by a piece of vitelline membrane seventh passage, 71 days after the beginning of the culture, 18 days after the separation from the mesonephros. × 40

Figure 22.—Histological section of tumor Z200 separated from the mesonephros by a piece of vitelline membrane eighth subculture, 76 days after the beginning of the culture, 22 days after isolation from the mesonephros. × 440



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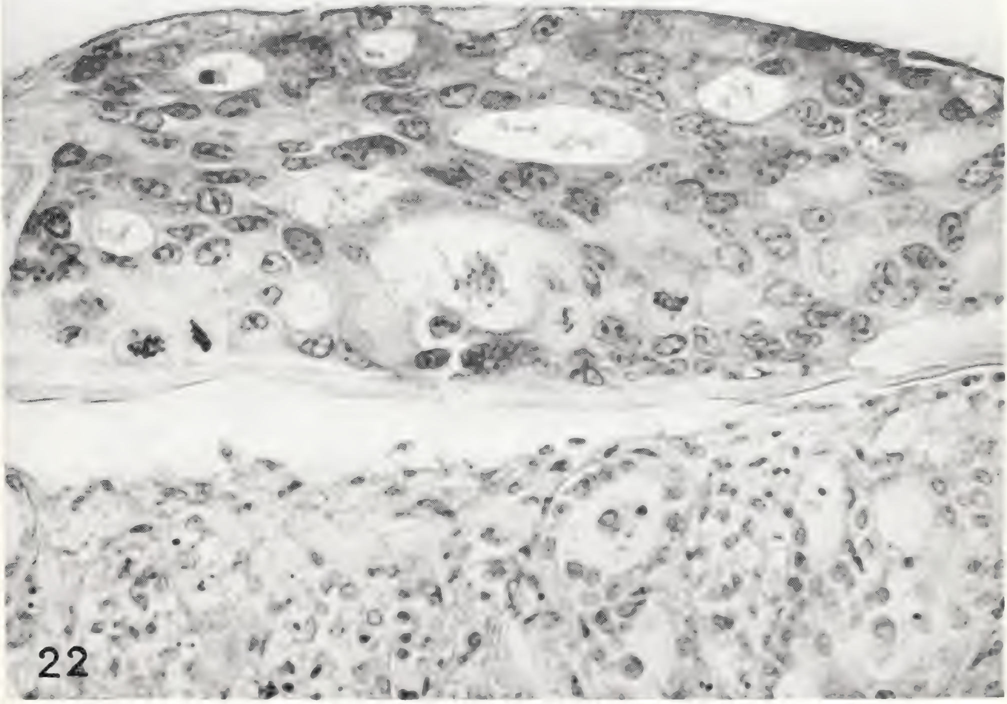

22

A TISSUE-CULTURE APPROACH TO DEMYELINATIVE DISORDERS[1,2]

MURRAY B. BORNSTEIN,[3] Laboratory of Cellular Neurophysiology, Department of Neurology, The Mount Sinai Hospital, New York, New York

SUMMARY

Myelinated cultures of rat and mouse cerebellum respond with a characteristic and specific demyelinating pattern when exposed to sera withdrawn from rabbits, guinea pigs, mice, and rats subjected to experimental "allergic" encephalomyelitis (EAE). The active factor(s) in EAE serum is an antibody(s). The reaction is also demonstrably complement-dependent. Fluorescence studies reveal EAE globulins associated with neuroglial cell membranes and cytoplasm and the myelin sheath. Identical patterns of *in vitro* demyelination follow the application of some sera from patients with demyelinative disease. Rarely, sera from normal or patient controls possess similar activity. The mechanism of the human factors includes a complement dependence. Further immunological studies are in progress. Demyelinated cultures of mammalian central nerve tissue possess the ability to remyelinate.—NCI Monogr. No. 11: 197–214, 1963.

AT THE annual meeting of the Tissue Culture Association 2 years ago, I described a characteristic and specific pattern of demyelination invariably produced in cultured fragments of rat cerebellum by the sera of rabbits subjected to experimental "allergic" encephalomyelitis (EAE). The reaction was never produced by normal or nephrotoxic rabbit sera (*1*). Moreover, the encephalotoxic sera had no effect on cultures of rat liver, kidney, muscle, or fibroblasts. It was also mentioned that demyelinated cultures would remyelinate after they were returned to their normal *in vitro* environment.

Since then, the sera of other animals have been studied for similar demyelinating factors and their immunological characteristics. In addition, the sera of patients with and without demyelinative diseases were tested to determine whether or not they might also possess demyelinative

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[1] Presented at The 13th Annual Meeting of the Tissue Culture Association, Washington, D.C., May 29–31, 1962.

[2] Supported by research grant B-1913 from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service, grant 246 from the National Multiple Sclerosis Society, and funds 139 and 861 from Department of Neurology, The Mount Sinai Hospital, New York, N.Y.

[3] Dr. Stanley H. Appel collaborated in the studies on experimental "allergic" encephalomyelitis. The author is grateful for the technical assistance of Miss Annelies Herrmann, Miss Rachel Monzain, Mrs. Barbara Nahmmacher, and Mr. Raul Nieves.

activity *in vitro*. Some of the results of these experiments will be presented today: 1) the tests of other EAE sera and the analyses for factors responsible for the *in vitro* demyelination; 2) the results of testing human sera; and 3) evidence that mammalian central nerve tissue has the ability to remyelinate *in vitro*.

MATERIALS AND METHODS

The method of culture of mammalian cerebellum in the Maximow slide assembly remains unchanged (*2*). The nutrient medium, however, has undergone some alterations. As a prelude to testing human sera, the human-placental-cord serum content of the normal feeding solution was replaced by either fetal bovine or horse serum. Since the chick embryonic extract was the source of occasional contamination by pleuropneumonia-like organisms (PPLO), it was eliminated entirely. Thus, the nutrient solution is now composed of 40 percent horse or fetal bovine serum, 25 percent bovine serum ultrafiltrate, and 35 percent Simms' balanced salt solution with glucose added to yield a final concentration of 6.0 g per liter.

EAE was induced in rabbits, guinea pigs, and rats by the intracutaneous inoculation of whole bovine or guinea-pig spinal cord plus Freund's adjuvant (*3*). Animal and human sera were stored at −70° C until used.

The immunological procedures used in the analysis of sera have been described (*4*) and will be mentioned at the appropriate times in the text.

Tests for the *in vitro* effects of human sera were performed in the same way as those with animal (EAE) sera (*1*). In brief, selected, well-myelinated, "sister" cultures were exposed in a random distribution to 1) feeding solution alone, 2) feeding solution plus normal human serum, 3) feeding solution plus a known demyelinating (EAE or human) serum, or 4) feeding solution plus an unknown serum. All sera were added in the same amounts. The known abnormal serum, an additional control, was included for 2 reasons: The presence of a nonspecific cytotoxic effect could be determined by comparison with the normal serum and the no-serum controls. Second, the reactivity to the specific encephalotoxic factors could be evaluated for that particular group of "sister" cultures. The test series was observed closely for 4 days after the addition of the various sera. The undoubted presence of the characteristic pattern of demyelination, as previously established by the EAE experiences, in the absence of a gross, general cytotoxic effect, was the essential criterion for designation of a particular sample of human serum as positive or demyelinating.

RESULTS

The pattern of demyelination was originally described as occurring in cultures of rat cerebellum exposed to rabbit EAE sera (*1*). The term "EAE serum," strictly speaking, is a misnomer, but is a convenient way to refer to serum obtained from an animal which 1) had received an EAE-producing inoculum, 2) had or would develop neurological symptoms such as weakness, paralysis, ataxia, sphincter disturbances, etc., and 3) eventually had the characteristic histological lesions of EAE demonstrated.

Briefly, the reaction begins as a swelling of neuroglial cells that indent or displace nearby axons or other structures (fig. 5). Soon, the myelin sheaths develop fusiform swellings (figs. 2 and 5) that may enlarge and, eventually, break away to lie free of the axon as myelin fragments (figs. 6, 7, and 10). Both myelin fragments and apparently intact sheaths are more or less rapidly reduced to scattered fat droplets (figs. 3, 7, and 11). Meanwhile, neuroglia have continued to swell and some die (figs. 3, 5, 7, and 10). Neuron somas (figs. 8 and 12) and axis cylinders, however, appear little affected by the demyelinating experience.

During the past 2 years, this characteristic reaction has been reproduced in cultures of rat cerebellum by the sera of many more rabbits. In addition, the sera of numerous guinea pigs, some rats, and mice with EAE have provoked the same reaction. No normal rabbit, guinea pig, rat, or mouse serum has had a similar effect. Moreover, cultures of mouse cerebellum undergo an identical demyelinating reaction (figs. 1, 2, and 3) when exposed to rabbit, guinea pig, or mouse EAE serum.

A detailed account of the analysis of EAE sera for immunological mechanisms will soon be published (*5*). However, some of the data are pertinent to this symposium and will be mentioned.

The destruction of complement components by exposure of active sera to 56° C or 0.15 N NH_4OH removes the *in vitro* demyelinative potency. Full activity is restored by the addition of fresh, normal serum.

The globulin fraction (obtained by salting out with NH_4SO_4), but not the albumin, produces the characteristic tissue-culture response in the presence of complement. Exposure of either EAE serum or its globulin component to bovine or rabbit spinal cord removes the active factor, whereas homologous or heterologous liver, lung, kidney, or red blood cells do not. These data strongly suggest the presence of a specific antibody.

Finally, the application of fluorescence antibody techniques reveals the EAE globulins located on neuroglial cell membranes and myelin sheaths, *i.e.*, modified neuroglial cell membranes (*6*), during the early stages of demyelination. Later, the globulins appear diffusely scattered throughout the neuroglial cytoplasm. The albumin of EAE sera and both the albumin and globulin of normal sera appear as punctate accumulations within the glia—a pattern previously described (*7*) and considered to be the result of pinocytosis.

The next step in this series of experiments was to determine whether or not the sera from patients with demyelinative disorders would reproduce the now familiar pattern of *in vitro* demyelination. Figures 4 through 7 demonstrate the identical pattern of demyelination after the application of a 50 percent concentration of serum withdrawn from a patient (V. D., #A-7321, Veterans Administration Hospital) during an acute exacerbation of long-standing multiple sclerosis.

Although no actual titrations have been attempted with either EAE or human sera, an estimate of potency may be based on the time required for demyelination to be completed (*1*). With this measure, most human sera are less potent than EAE sera since a 50 percent concentration may be needed to equal the effect consistently produced by 20 percent of EAE serum. Since 50 percent human serum was the maximum introduced for testing, some borderline cases may be called negative. The general cytotoxic effect which may be associated with higher concentrations of serum would, however, render the determination of a specific demyelinating action virtually impossible. It was thought better, therefore, to err on the side of conservatism.

Table 1 lists the results of tests of the first 105 sera obtained from patients and normal controls. I saw all of the patients whose sera were tested and examined many of them. It is not possible to present here the detailed descriptions of these patients. For purposes of classification, they have been divided into 3 categories: 1) *Demyelinative:* those diagnosed as having one or other of the known demyelinative diseases on the basis of history and clinical examination. The "multiple sclerosis: ? activity" patients are those with long-standing, severely crippling disease in whom manifestations of recent activity are not clearly evident. 2) *Nondemyelinative:* those diagnosed as having a disease of neoplastic, vascular, heredodegenerative or other etiology of destructive but not essentially demyelinative nature as determined by clinical laboratory tests or surgery. 3) *Unknown etiology:* those who enter with an acute first or second neurological episode possibly of a demyelinative disorder, but for which no etiological diagnosis can be proposed. Whether or not the third group should be included at this time can be debated, but the few data are available and the patients are, clearly, of major interest.

The same immunological methods used for the study of the EAE sera are being applied to an analysis of the active human sera. Preliminary results suggest that the human factors are also complement-dependent,

TABLE 1.—Demyelinating action of human sera on cultured rat cerebellum

Clinical diagnosis	Positive	Negative
Demyelinative:		
Multiple sclerosis: active (exacerbation)	14	6
Multiple sclerosis: ? activity	3	1
Multiple sclerosis: inactive (remission)	0	15
Neuromyelitis optica	1	0
Optic neuritis	1	2
Amyotrophic lateral sclerosis	1	2
Postvaccinal encephalomyelitis: acute	1	0
Postvaccinal encephalomyelitis: chronic	0	1
Nondemyelinative:		
Normal controls	1	16
Central nervous system disease, acute and chronic	0	18
Neoplasms of central nervous system	0	2
Neoplasms of central nervous system treated with CO^{60}	2	1
Unknown etiology:	5	11

since heating to 56° C for ½ hour destroys, and the addition of fresh, normal human serum restores their potency.

A totally demyelinated fragment is by no means totally destroyed. Most neurons and their axis cylinders are demonstrably intact (*1*) and many viable neuroglia remain. If the cell population has not become too small, *i.e.*, if a dense community of neuroglia still intimately surrounds neurons and axons, the fragments will remyelinate after having been washed free of the demyelinating mixture and returned to their normal nutrient environment. As long as the adequate community exists, cultures may be repeatedly de- and re-myelinated. Indeed, one group of rat fragments successfully completed an *in vitro* myelination and 3 cycles of demyelination and remyelination during their 60 days in culture. Fortuitous conditions occasionally permit the additional observation that not only does tissue of the central nervous system possess remyelinating ability, but also that particular axons may regain their myelin sheaths (figs. 9 through 13). One further observation, which may be surmised from the figures, is that remyelination is frequently incomplete. The thickness of the sheath may not attain that shown by the sister control cultures. In addition, particular axons may not be ensheathed along their entire length, but, rather, in segments.

DISCUSSION

The interpretation of tissue-culture techniques applied to the study of specific immunological phenomena and their potentially related disease processes must be viewed with considerable skepticism in the light of the general cytotoxic properties displayed by almost all sera. Even with as favorable a reactive object as the myelinated cultures, translation of the results into terms of *in vivo* disease processes should be attempted with caution. Nevertheless, the striking similarities between these findings and those well established by previous *in vivo* studies of EAE (*8*)—the *in vitro* reproduction of the fundamental fact of the disorder, *i.e.*, demyelination, its histopathological resemblance to the lesion in the animal, and its apparent adherence to the organ specificity *in vitro*—support the faithfulness of the system.

Specific circulating antibody(s) is the factor responsible for the specific *in vitro* effect of EAE sera. This fact cannot, by itself, be applied to an understanding of the animal disorder, but neither can it be dismissed in any serious consideration. Mutually exploratory animal and tissue-culture experiments may gain the required balanced understanding of the various cellular, circulating, genetic, and other factors participating in the process.

Some human sera produce a pattern of *in vitro* demyelination indistinguishable from that caused by EAE sera. The occurrence of the *in vitro* phenomenon appears to be significantly related to patients who are experiencing an active, demyelinative episode. However, a possible etiologic or pathogenetic relationship to the disease process itself remains undetermined and should not be inferred from the data. The results mean only that a system is available to test many more sera of patients and controls, to follow particular patients through the variations of their clinical courses, and to analyze the positive sera for mechanisms of action. Eventually enough data may be acquired to permit a significant statement concerning the human demyelinative diseases.

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(*8*) Kies, M. W., and Alvord, E. C., Jr.: "Allergic" Encephalomyelitis. Springfield, Illinois, Charles C Thomas, 1959.

PLATES

All photographs are of living, unstained cultures of rat or mouse cerebellum taken with ordinary bright-field illumination at a magnification of 600 diameters. Except for inserts of neuron somas, all pictures in each series represent the same area of a particular culture. Scale at lower border of each plate represents 50 μ.

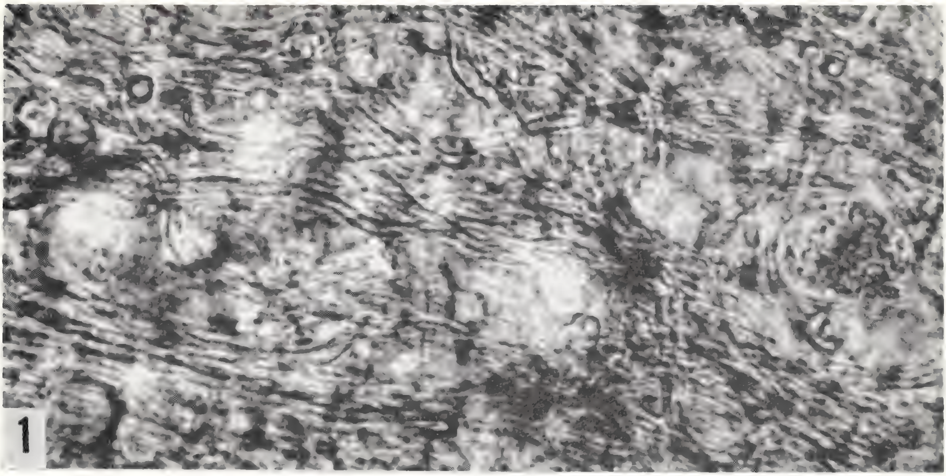
PLATE 30

Figures 1 through 3 represent a culture of mouse cerebellum, M48/3, 19 days *in vitro,* before and after exposure to a 20 percent concentration of guinea pig EAE serum.

FIGURE 1.—Control period, normal myelinated axons.

FIGURE 2.—After 4 hours' exposure to EAE serum, myelin sheaths display a number of fusiform swellings.

FIGURE 3.—After 10 hours' exposure to EAE serum, the culture is totally demyelinated. A single, enormously swollen neuroglial cell occupies a large part of photograph (*arrow* G).



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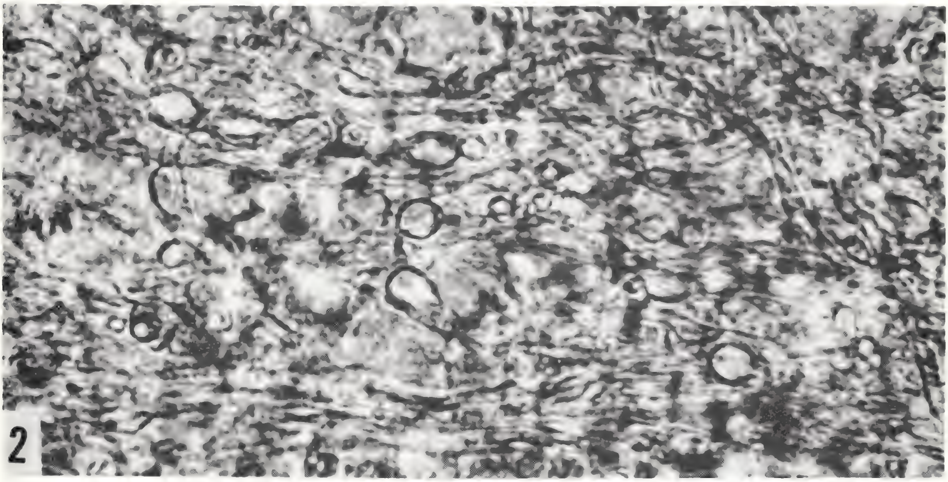

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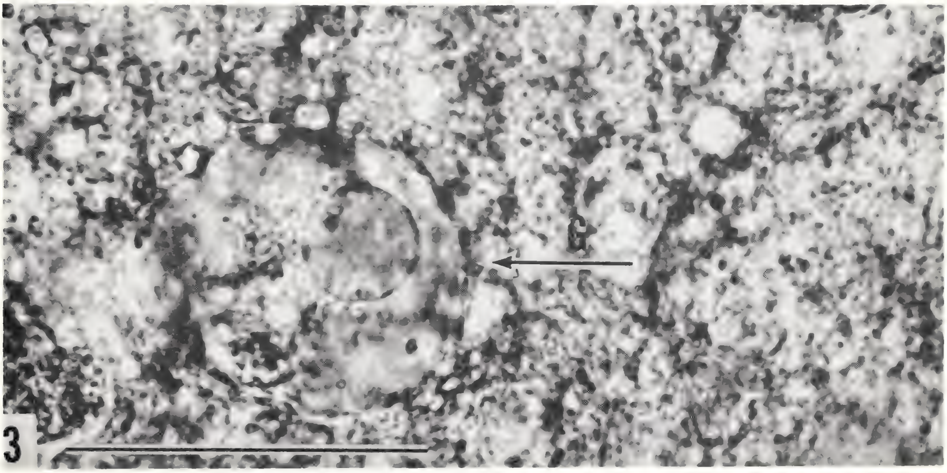

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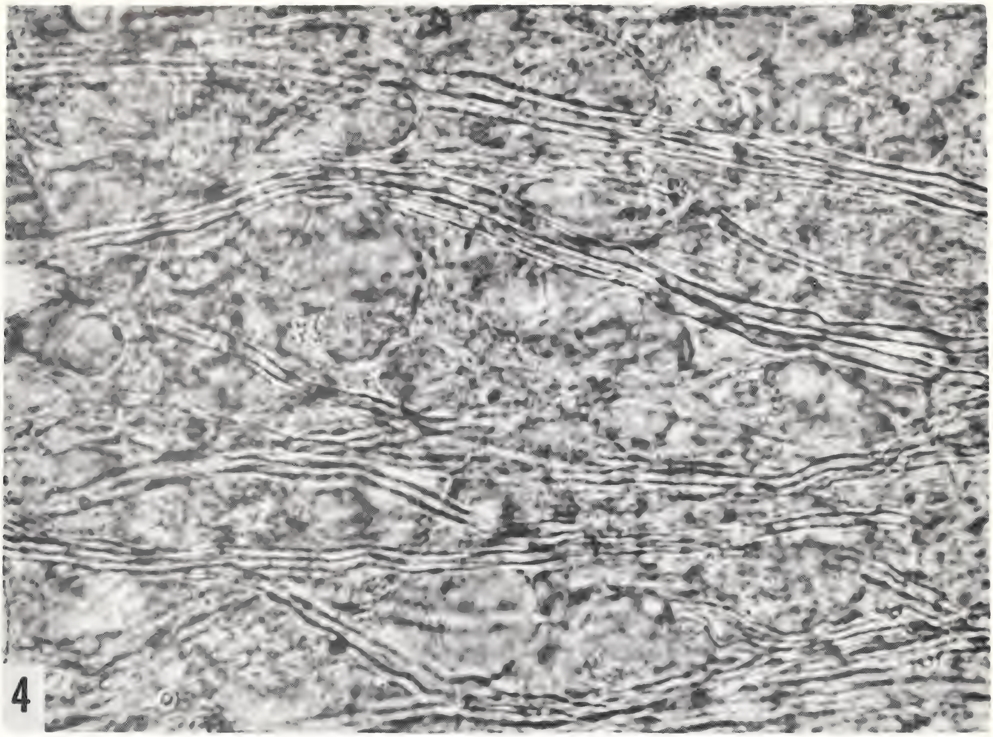
BORNSTEIN

PLATE 31

Figures 4 through 8 represent demyelination produced in a culture of rat cerebellum, R62/1, 21 days *in vitro*, by the exposure to a 50 percent concentration of serum from a patient with a demyelinative disease.

FIGURE 4.—Control, before exposure. Normal myelinated axons.

FIGURE 5.—Twenty minutes after the application of serum. *Note* distortions and displacements of myelinated axons (*arrows* D) produced by swelling neuroglial cells (*arrows* G).



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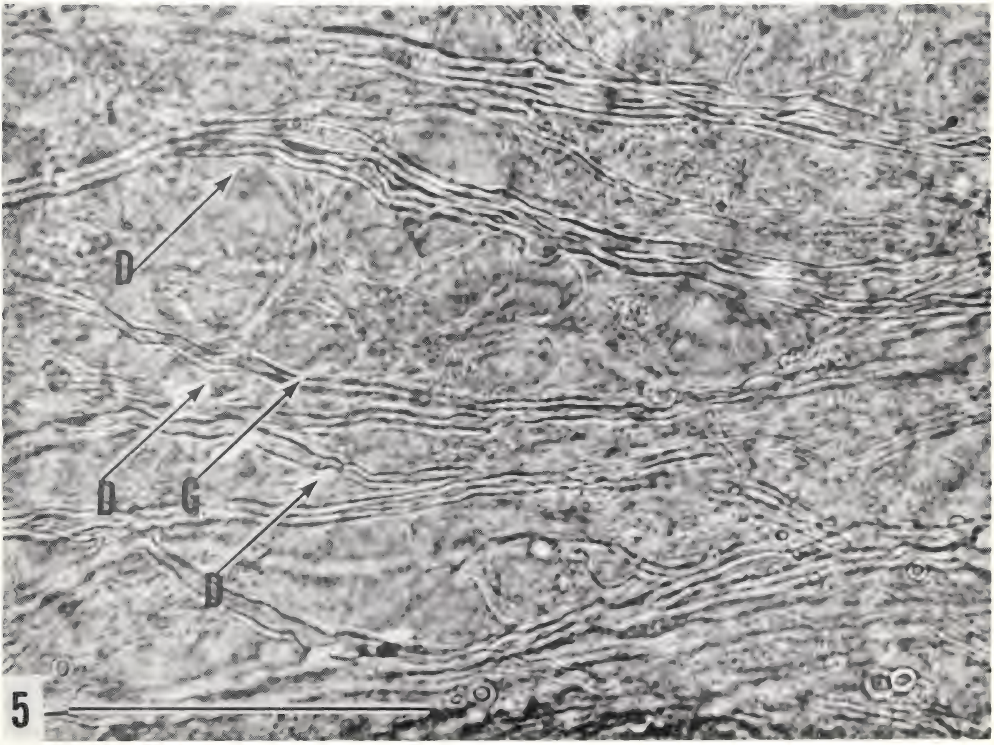

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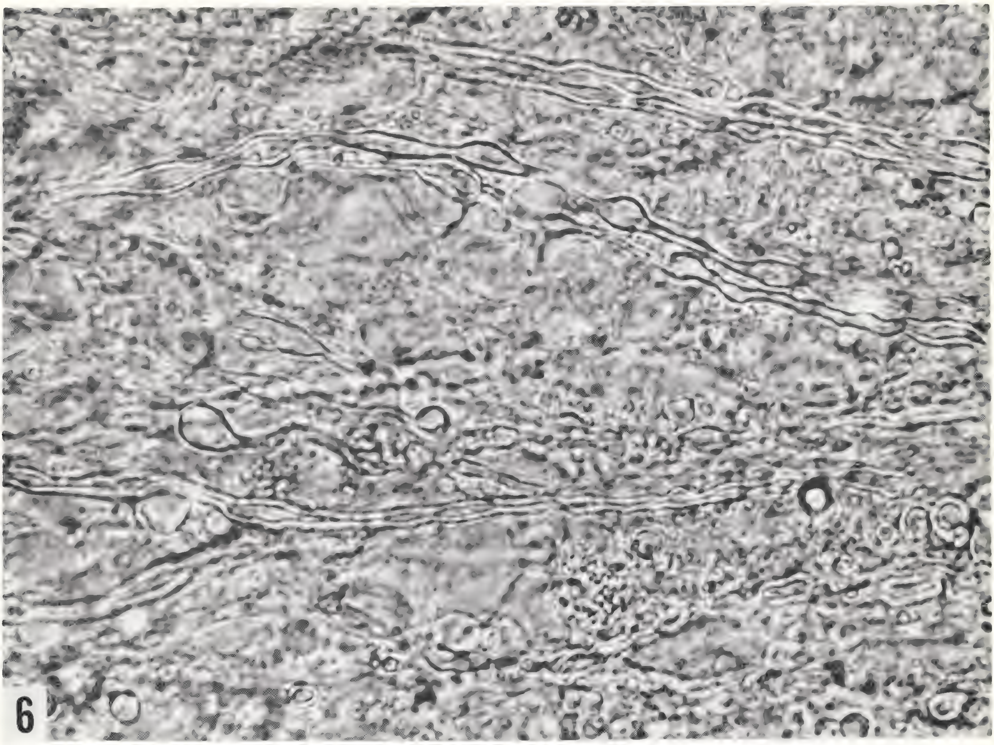
PLATE 32

FIGURE 6.—After 4 hours' exposure to human serum. All myelin sheaths affected. Some display fusiform swellings, but many have already become disrupted. Myelin fragments lie free of axons.

FIGURE 7.—After 8 hours' exposure to human serum. Most myelin sheaths have broken down into fragments. Myelin is already being reduced to small fat droplets. A large, swollen neuroglial cell is designated by *arrow*.

FIGURE 8.—After 8 hours' exposure to human serum. A different area of same culture showing relatively unaffected neuron somas.



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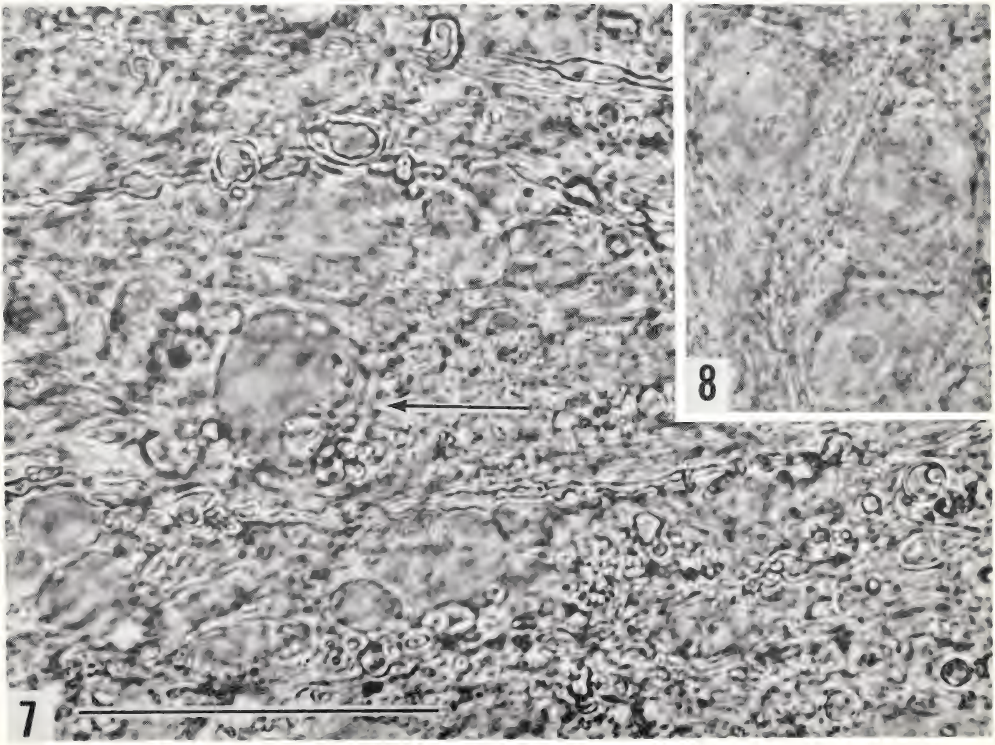

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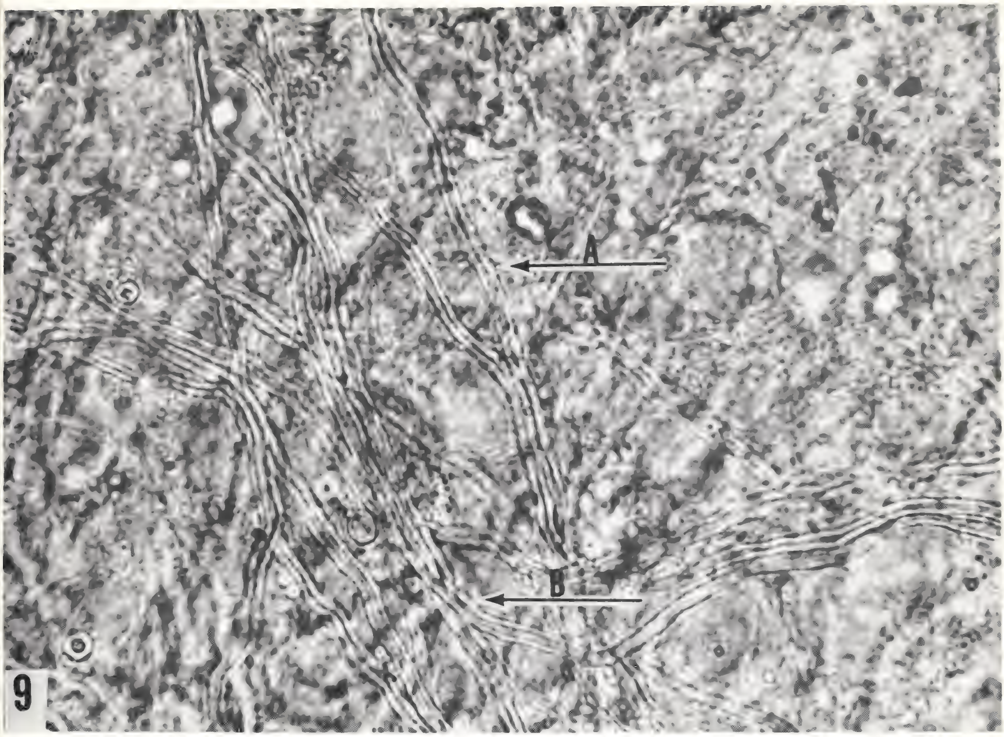
Plate 33

Figures 9 through 13 represent a culture of rat cerebellum, R34/10, demyelinated on the 16th day *in vitro,* by exposure to a 20 percent concentration of rabbit EAE serum, washed free of rabbit serum on the 17th day *in vitro,* and then followed during its period of remyelination.

Figure 9.—Control period demonstrating normal, healthy myelinated axons. *Arrows* A and B designate axons that will remyelinate (*cf* fig. 13). Others may also be found with some study of the 2 illustrations.

Figure 10.—After 4½ hours' exposure to rabbit EAE serum. All myelin sheaths have disappeared leaving some fragments undergoing lipolysis. *Arrows* G designate swollen neuroglia.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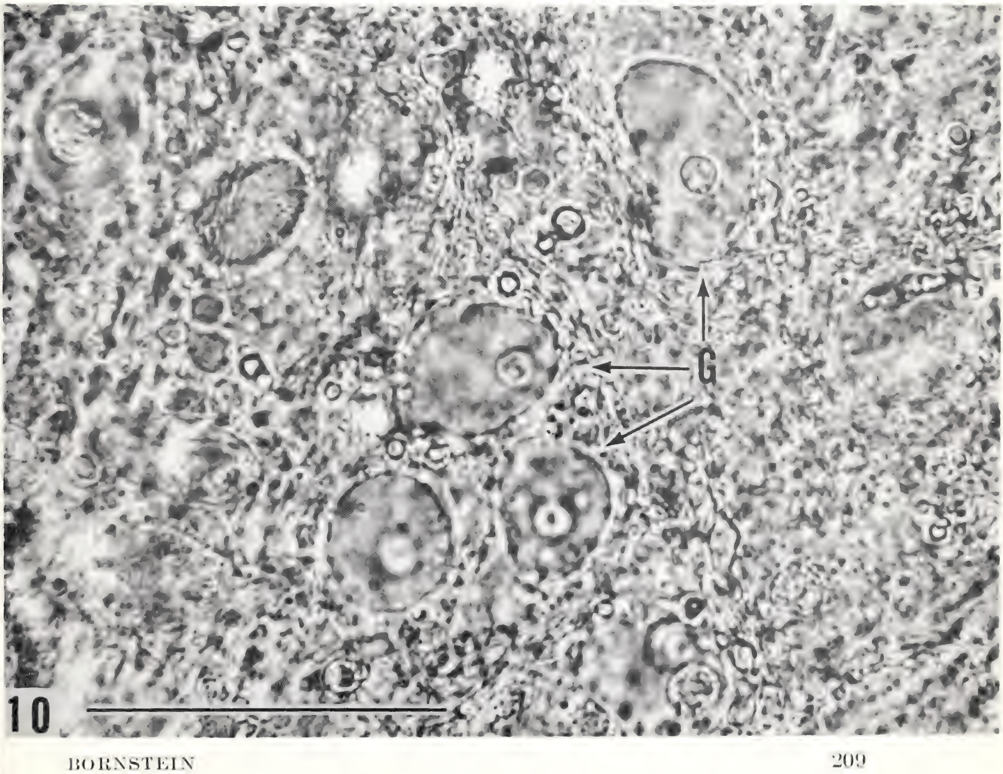
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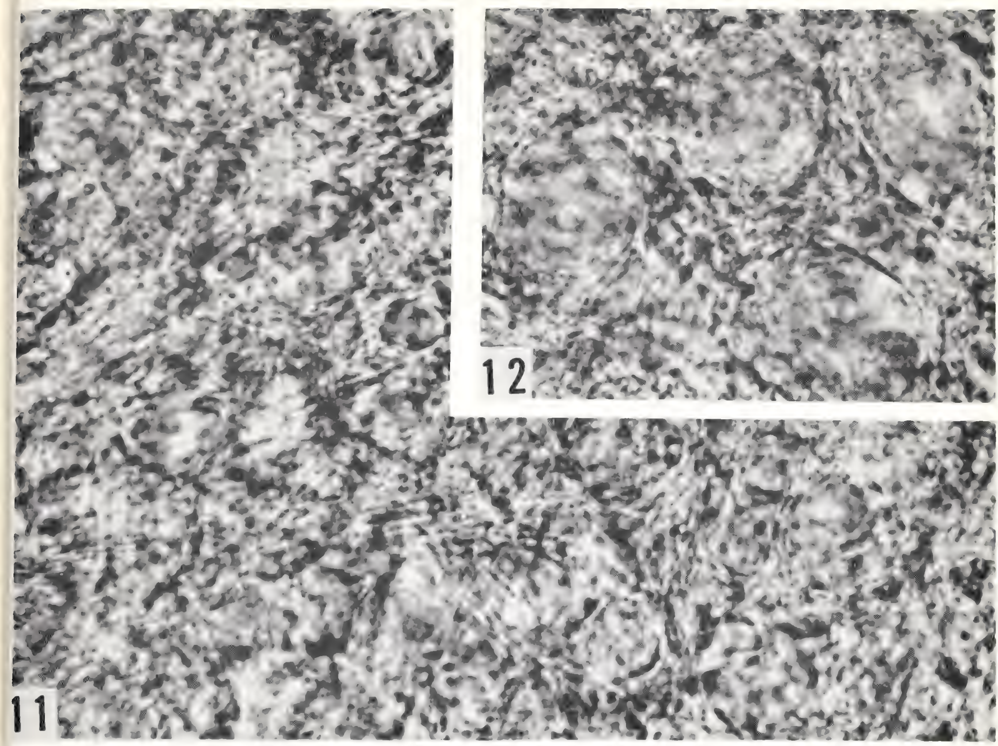
Plate 34

Figure 11.—One day after total demyelination produced by 24-hour exposure to rabbit EAE serum. Returned to normal nutrient medium. *Note* disappearance of dead neuroglia, scattered fat droplets, and the difficulty with which denuded axons may be visualized against the neuroglial background.

Figure 12.—Same time as figure 11 but a different region of the culture to show the relatively normal, unaffected neuron somas.

Figure 13.—Twelve days after demyelination and return to normal *in vitro* conditions. Remyelinated axons, including A and B, as seen in figure 9 (16 days *in vitro*) slightly displaced by previous nurologlial swellings and rearrangements.



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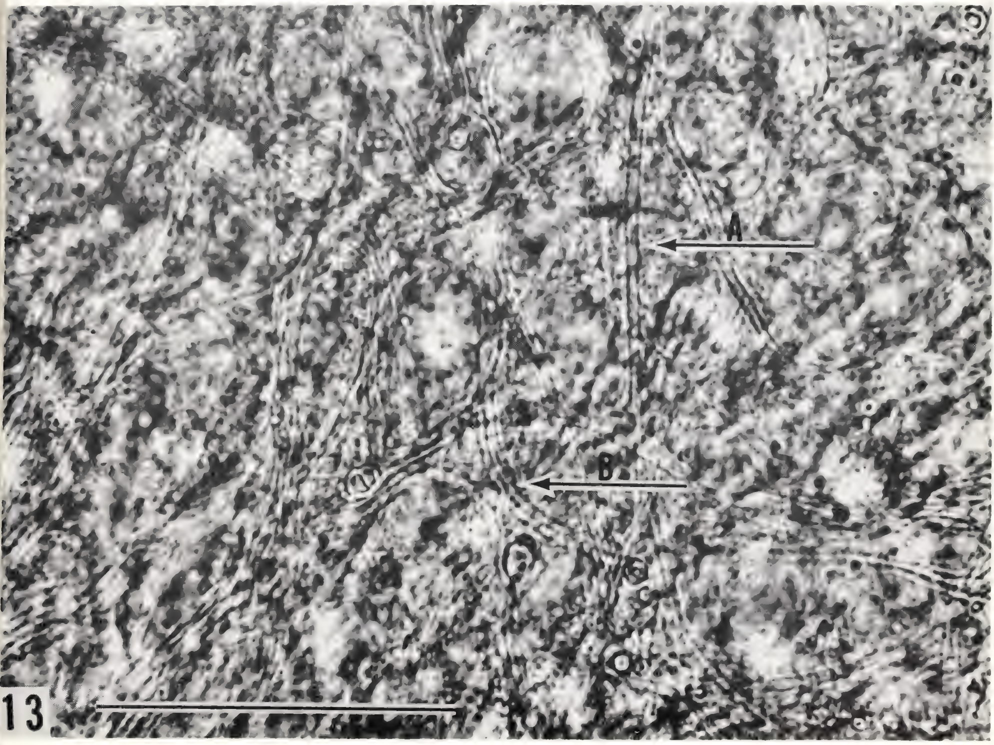

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DISCUSSION

Serum Factors in Experimental Allergic Encephalomyelitis and Neurological Diseases of Man.[1] PHILIP Y. PATERSON,[2] Department of Medicine, New York University School of Medicine, New York, New York

I have followed the work of Dr. Bornstein and Dr. Appel with interest. This work is an excellent example of the enormous dividends often realized by application of new techniques to long-standing problems—the use of myelinating brain-tissue cultures in the study of the pathogenesis of allergic encephalomyelitis and its relationship to neurological disease of man.

Their study has provided new insight into the pathogenesis of allergic encephalomyelitis (AE). Although AE is believed to have an immunological mechanism, the type of immune response causing the disease has eluded precise definition (*1, 2*). A major question has been as to whether the perivascular inflammation and demyelination characterizing AE is caused by cell-bound immune factors, associated with delayed-type hypersensitivity to brain, or whether the tissue damage is caused by classical serum antibody (*3*). The transfer of AE in rats by the use of lymph-node cells of spinal cord-sensitized donors has not settled this question. For in these transfers (*2*), it is impossible to conclude whether the lesions of AE in recipient animals were caused by conventional antibodies produced and released by the donor cells after their transfer or directly by the cells themselves.

Rabbits and other animals sensitized to nervous tissue plus adjuvant are known to produce serum antibodies that specifically fix complement with nervous tissue *in vitro*. These complement-fixing (CF) antibrain antibodies, however, have been shown to have little, if any, role in causing AE. For example, the CF antibodies appear to be directed against antigens in nervous tissue which are similar to but distinct from antigens concerned in AE (*4*). No direct relationship exists between the presence of CF antibody and occurrence of AE in individual sensitized animals (*5, 6*). The strongest argument against the concept that these or any circulating antibody cause AE has been the unsuccessful attempts at passive transfer of AE to normal animals with immune serum [reviewed by Chase (*7*)].

The serum factor in rabbit sera described by Bornstein appears to be an antibody having cytotoxic activity directed against myelinated nerve fibers and glial cells in nervous tissue. An important question is whether this serum factor is or is not CF antibrain antibody. If the cytotoxic factor is different from CF antibody, it is a new type of antibody in AE.

An answer to this question was obtained as follows: Dr. Bornstein's laboratory provided us with rabbit immune sera that his group had already assayed for demyelinating activity in rat brain cultures. We assayed these sera for content of CF anti-rat brain antibody in our laboratory using a precise, quantitative technique (*2*).

A summary of comparative assays on 15 sera from 10 rabbits is shown in table 1. Seven sera were positive for CF antibody and negative for demyelinating activity. Six sera were positive in both respects. One serum had no CF antibody but clearly possessed demyelinating activity. One serum was negative in both respects.

These data (table 1) indicate that the cytotoxic antibody and CF antibrain antibodies represent independent or different types of immune responses to nervous tissue. Whether the cytotoxic antibody is *the* factor or *a* factor responsible for AE is not yet clear. The strong possibility exists that it may indeed be an important factor. That this antibody causes fragmentation of myelin further supports the view that antigens implicated in AE reside in myelin and/or cells concerned with myelin. A major step in direct support of a causal role for the cytotoxic antibody would be the transfer of

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[1] The complement-fixation studies performed by Miss Elaine M. Coia in collaboration with Dr. Murray B. Bornstein and Dr. S. Appel were supported by grant B-3104, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Service.

[2] Recipient of Investigatorship of Health Research Council of City of New York.

TABLE 1.—Summary of comparative assays of complement-fixing (CF) antibrain antibody and demyelinating activity in rabbit sera against rat brain

Number of sera assayed*	CF antibrain antibody †	Demyelinating activity ‡
7	+	0
6	+	+
1	0	+
1	0	0

* Four sera obtained from 1 rabbit 2 days to approximately 2 months after last foot pad injection of homologous spinal cord plus adjuvant. Remaining 11 sera obtained from 9 rabbits usually 2 weeks after last intraperitoneal injection of bovine brain plus adjuvant with or without mycobacteria.

† Presence of antibody indicated by + and indicative of more than 5 – 50 percent hemolytic units of complement (C′H50), fixed specifically with ethanol extract of lyophilized rat brain used as antigen.

‡ Presence of demyelinating activity indicated by + and indicates fragmentation and dissolution of myelin within 96 hours or less after addition of serum to rat brain tissue culture.

AE to normal animals with cytotoxic antibody. This step has yet to be accomplished and may prove difficult. For example, transferred cytotoxic antibody after dilution in recipient animals may be unable to diffuse through the blood-brain-barrier by passive means in sufficient amounts to combine with brain antigen and cause detectable tissue damage.

These findings unquestionably indicate the need for intensifying investigation of conventional serum antibodies, as well as of "sensitized" cells, in AE. The results of such investigations may well hold the key to unlocking the riddle of AE.

It is of special interest to us that immune sera containing CF antibrain antibody do not exert a cytotoxic effect on living brain cultures. Work in our laboratory suggests that the CF antibodies exert a protective effect in AE *in vivo* (*3*, *8*): Passively administered immune serum containing CF antibodies may suppress or prevent development of AE in actively sensitized rats. It has been suggested that the CF antibodies may act as "blocking antibodies" and, by harmlessly combining with antigenic sites in nervous tissue, interfere with the development of AE (*3*). The lack of cytotoxicity of CF antibody in brain cultures is consistent with this thesis.

For many years AE has been considered to represent a meaningful experimental model for the study of certain neurological diseases of man. There has been much debate as to whether AE actually is the equivalent of such diseases as postvaccinal encephalitis or multiple sclerosis; in 1959 a symposium was devoted to this issue (*9*). Bornstein found that sera of patients with active neurological disorders exhibit effects in brain cultures indistinguishable from those seen with AE sera. This observation certainly is another link in the chain of evidence that AE and demyelinating diseases of man may be closely related. The cytotoxic serum factor in patients with multiple sclerosis is an exciting finding though it should be stressed that its role is not yet defined. It is possible that it may prove a key finding in the continuing struggle to unravel the pathogenesis of multiple sclerosis and provide a means of devising control over this and related neurological diseases.

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ACTION OF SEX HORMONES ON DEPENDENT TUMORS IN CELL AND ORGAN-CULTURE SYSTEMS[1, 2]

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SUMMARY

Three sex hormone-dependent hamster tumors have been investigated in dispersed cell and organ-culture systems. Tumor cells emigrate from primary explants and established outgrowths, exhibit growth (mitosis), and may be passaged serially in hormone-free medium. Incorporation of hormones into the culture medium, either initially or after an outgrowth is already established, fails to elicit any deflection from the patterns of behavior observed in untreated cultures. Cultures of a renal carcinoma and a trichoepithelioma, when reimplanted into animals, have grown only in the presence of appropriate exogenous hormones. In organotypic culture, behavior of these tumor tissues with respect to hormones is unlike that of cell cultures. The renal carcinoma and a leiomyoma rapidly deteriorate in hormone-free media; the trichoepithelioma, however, shows appreciable survival after 1 month in hormone-free organ culture, but without mitoses. In the presence of hormone(s) all 3 tumors show enhanced survival and mitoses occur. The capacity of these neoplasms to respond to hormonal excitation appears to be related to tissue integrity. If the architectural pattern of the tumors is destroyed, as in dispersed cell culture, a state of reversible "autonomy" seems to be achieved. Direct versus indirect hormone-tumor cell relationships in these neoplasms are discussed and a brief description is given of these tumors *in vivo*.—NCI Monogr. No. 11: 215–226, 1963.

THAT HORMONES might be implicated in carcinogenesis was recognized during the first decade of the present century (*1*). For some 30 years (*2*) endocrine carcinogenesis has constituted an important segment of cancer research at both the basic and clinical levels. Although other hormones have been investigated, by far the greatest attention has been focused on the sex steroids, particularly the estrogens. There is wide acceptance of the fact that disturbances in estrogen balance are associated

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[3] With the assistance of Miss Sumiko Sakaguchi and Mrs. Alice Dodge. Sincere appreciation is extended to Professor Hadley Kirkman for making his dependent tumor lines available to us and for his many provocative and helpful suggestions.

with the production and growth control of a variety of neoplasms; however, there seems to be no general agreement concerning the precise role of the hormones. Despite the quite convincing evidence that estrogens are directly responsible for carcinoma of the hamster kidney (*3, 4*) and that they seem to be directly concerned in some rodent mammary-gland tumors (*5, 6*), Pincus (*7*), in a recent review, struck a conservative note regarding the implication of estrogen as a direct carcinogen, reminding us that, ". . . hormones do not create processes *de novo,* [but] . . . act as regulators and permissive agents."

Even less is known about the effectiveness of androgen to initiate abnormal growth (*8, 9*) though its profound influence on prostatic carcinoma has long been recognized (*10*).

Because of the complexities of whole animal experimentation, tissue- and organ-culture systems seem to offer, *a priori,* a reasonable approach toward a better resolution of some aspects of hormone-tumor interplay. It is my intention to review briefly the characteristics, *in vivo,* of 3 sex hormone-induced, dependent tumors in the golden hamster, all of which were initially described by Kirkman (*11, 12*), and to comment on the behavior of each tumor in cell and organ culture.

OBSERVATIONS

Renal Carcinoma

In Vivo

Nearly 100 percent of all male or gonadectomized hamsters when treated with estrogens, including diethylstilbestrol, ultimately develop carcinomas of the kidneys. Primary tumors transplant readily to estrogenized hosts and progress slowly toward autonomy. Estrogen withdrawal results in rapid involution of primary tumors as well as of dependent transplants. Antiestrogens, *e.g.*, deoxycorticosterone and progesterone, tend to inhibit induction and retard or prevent transplant growth. Androgens block the induction of primary tumors, but do not retard transplant growth.

In Vitro

Cell culture [4]

Tissue explanted from primary tumors, transplants, or pulmonary metastases to a thin film of cock plasma or rat-tail collagen overlaid with medium #199 supplemented with 5 to 10 percent chick EE50 gives rise, especially after pretreatment with tryspin and/or collagenase (*13*), to epithelioid sheets in which mitotic figures may be observed. The addition of estrogen to the medium, even in heavy concentrations, fails to alter visibly the outgrowth pattern or the frequency of cell division or survival time (*14*).

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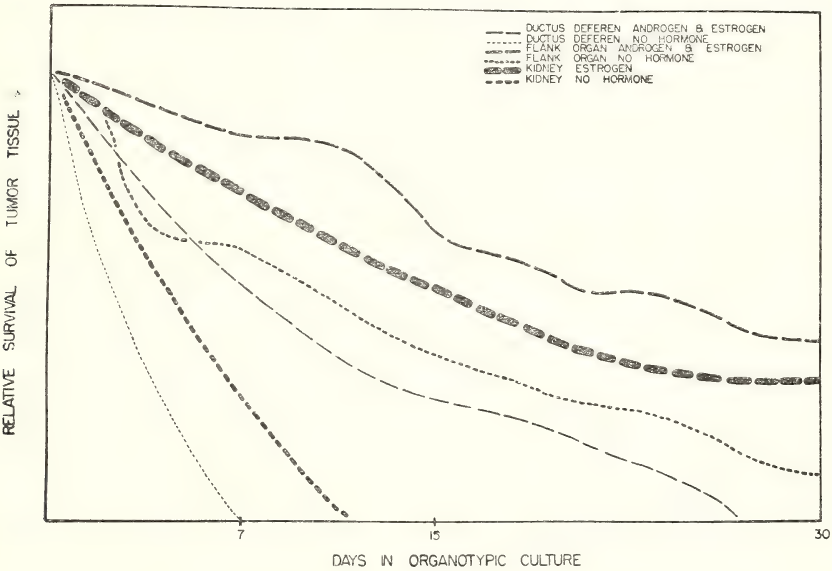
[4] Dontenwill and Wrba (*15*) have studied the effects of estrogen on roller-tube cultures of this tumor and believe that the hormone prolongs viability of the primary explant and exerts some influence on the cellular outgrowth. The great difficulty of drawing any conclusions from similar studies was largely responsible for our turning to the technique of enzymatic dispersion of this tumor tissue (*14*).

Organ culture

Organotypic cultures of renal carcinoma show relatively good survival and some growth (mitoses) only when estrogen is incorporated into the culture medium. In the absence of estrogen, the tumor tissue shows necrotic changes within the 1st week and complete failure to survive beyond the end of the 2d week (text-fig. 1). On the other hand, incorporation of 5, 10, or 25 µg per ml of estrogen (diethylstilbestrol) produces a burst of mitotic activity during the 2d week of culture and prolongs survival to at least 1 month (*14*).



Text-figure 1.—Subjective graphic representation of effect of hormone(s) on dependent hamster tumors in organotypic cultures derived from semiquantitative data.

Flank-Organ Trichoepithelioma [5]

In Vivo

The flank-organ tumor was induced initially by Kirkman during a study of the efficacy of testosterone to inhibit the carcinogenic effect of estrogens on the hamster kidney (*12*). Long-term (200–400 days) treatment of either sex with simultaneously implanted pellets of estrogen and testosterone produces large, palpable masses, usually bilaterally, in the costovertebral scent gland (flank organ). Estrogen alone, in pellet administration, fails to produce tumors; in fact, it tends to depress the entire organ. Testosterone, however, never fails to induce a population of microscopic tumors that do not become palpable unless estrogen is added to the treatment. Recent detailed studies (unpublished) reveal that all old, untreated males bear definitely related preneoplastic lesions having characteristic morphology, and arising from flank-organ hair follicles. These preneoplastic nodules can be induced precociously in both young males and females as well as in castrates and hypophysectomized animals of either sex.

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[5] A comprehensive review of the biology of this tumor is currently being prepared by Kirkman and Algard.

In summary, this tumor has three distinct stages:

Stage I—a preneoplastic derivative of the hair bulb, normal in old males and inducible in young males and females with exogenous androgen alone.

Stage II—a microscopic or barely visible but nonpalpable tumor deriving from Stage I by growth, histological alteration, and/or fusions under the continuous administration of androgen.

Stage III—a large, palpable mass deriving from Stage II tumors under the combined action of androgen and estrogen.

Stage III tumors transplant readily to androgen-estrogen-treated hosts, grow slowly, metastasize frequently, and, upon hormone withdrawal, cease to grow but involute only slowly. As reported for interstitial-cell tumors in mice (*16*), even after several months in an untreated host nonpalpable transplants grow soon after administration of androgen-estrogen.

In Vitro

Cell culture

Primary tumor (stage III), transplants, and metastases of this trichoepithelioma, when minced and subjected to digestion with trypsin and/or collagenase and cultured on a thin clot with or without hormones in the feeding medium, yield an outgrowth pattern of the epithelioid type (*14*, *17*). Appearance of individual cells, colonies of cells, number of division figures observable, and survival time in culture are not significantly different in medium containing 0, 5, or 10 μg per ml of androgen-estrogen. Twenty-five μg per ml of androgen appeared to be toxic.

Organ culture

Although hormone-treated fragments of this tumor, in organotypic cultures, show enhanced survival and mitotic activity (peaking late in the 2d week of culture), untreated cultures also frequently show appreciable quantities of surviving tissue after 1 month in culture; however, cell division fails to occur in such non-hormone-treated cultures (*17*) (text-figs. 1 and 2).

Leiomyoma of the Ductus Deferens [6]

In Vivo

A high proportion of hamsters under long-term (300–400 days) continuous treatment with androgen-estrogen develop, in addition to the flank-organ tumor, leiomyomas of the ductus deferens and/or epididymal

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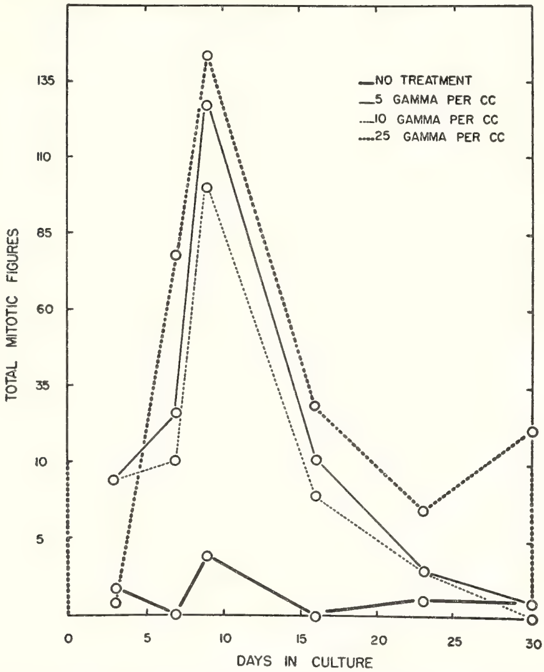
[6] A more detailed account of the characteristics of this tumor *in vitro* will be submitted in the near future.



TEXT-FIGURE 2.—Comparison of mitotic activity observed in untreated and androgen-estrogen-treated organ cultures of flank-organ epithelioma. (*Note* that lower part of graph is expanded.)

tail, or of the uterus. The ductus deferens tumor is moderately fast growing and is transplantable only to hosts receiving both inducing hormones. Metastasis to the lungs from transplants is not uncommon. Regression occurs rapidly following hormone withdrawal. There has, as yet, been no indication that this tumor has made any progress toward autonomy.

In Vitro

Cell culture

Cellular emigration from primary explants of the ductus deferens tumor in conventional Maximow or roller-tube cultures is relatively slow, but usually within a week or 10 days quite extensive outgrowths occur. Rate of emigration, outgrowth pattern, and the rate of cell division are not altered by the addition of 5 to 10 μg per ml of hormone mixture, nor is the culture survival time increased. Twenty-five μg per ml doses of combined hormones tend to inhibit outgrowth and to damage already established outgrowths; in addition, clot lysis is frequently several fold that observed in cultures receiving less or no hormones.

Organ culture

The demise of untreated organotypic cultures of this leiomyoma is rapid, severe deterioration occurring within 2 or 3 days and complete failure usual within 1 week (text-fig. 1). Survival in 5 and 10 μg of hormone mixture per ml of feeding medium is still quite good after 2 weeks, but typically, survival by the end of 1 month is erratic; no quantitative dose-survival relationship appears to exist. Survival is less in 25 μg per ml hormone doses.

DISCUSSION

The finding that 3 experimentally produced hormone-dependent hamster tumors behave differently with respect to hormone(s) *in vitro*, according to whether cells are encouraged to disperse or are forced to remain within the confines of an organotypic culture, raises more questions than it answers. The ability of the dependent cell to survive, move about, and divide in a hormone-free environment suggests that there is no strictly cellular requirement for hormone(s), *e.g.*, nutritional or metabolic, and tends to support the concept that exogenous hormones, *in vivo*, may well be permissive, interfering with "regulators" or "checks and balances" presumed to be ordinarily operative in maintaining a normal (non-neoplastic) state of affairs. Such controls are completely lacking in the cell culture milieu. This must remain a mere suggestion, since at the present moment we have to reckon with at least two complicating factors: 1) As pointed out by Franks and Barton (*18*), the intracellular organization of cells in cultures is quite disrupted, a fact which may not be insignificant in the interpretation of an "escape" from hormone senstitivity; 2) it is possible that the dependent tumor as it is taken from the animal actually contains a mixture of cells ranging from thoroughly dependent to at least partially autonomous and that the conditions of cell culture select for the latter. The rather limited study of culture reimplantation into animals argues against such an encouragement of autonomy in cell culture, since takes, thus far, of the renal and flank-organ tumors have occurred only in treated animals. An attempt to learn more about the effects of repeated cycling of a dependent tumor between animal and cell culture on progression from dependency to autonomy is now being made.

That the pattern of dependency found, *in vivo*, is reflected in organotypic culture may mean, as a corollary of 1), that there is less or no intracellular disorganization and therefore no loss of dependency. Fine-structure studies of such cultures should be made; again, however, Franks' investigation of mouse prostate (*18*) in testosterone-containing medium indicates that normal ultrastructure can be maintained in organotypic culture. That the maintenance of tumor architecture, *e.g.*, characteristic arrangement of tumor cells in relationship to supporting connective tissues, may be of cardinal significance in the retention of dependency in these organ cultures has been discussed elsewhere (*14*, *17*) and needs only be underscored at this time.

The precise role of hormones in this particular study of dependency remains enigmatic; however, it seems reasonable to assume that dependency is at the tissue level. To this extent hormonal influences seem to be of a direct nature. Whether or not these influences are "permissive" remains an open question.

The unique androgen-associated preneoplastic phase (stage I) described for the flank-organ trichoepithelioma may be of value in the determination of whether or not a hormone can exert a direct carcinogenic action at the tissue level. We have lately been comparing the growth and differentiation of prenatal and neonatal flank organ *in situ* with that in organotypic culture. It has been established that, during a week in culture, differentiation more or less parallels that seen *in situ*, *e.g.*, hair follicles develop and sebaceous glands appear. If this system can be coaxed to endure for a month in the presence of incorporated androgen and if androgen does act directly upon hair matrix as a carcinogen, stage I nodules might appear, or at least certain recognizable changes in the hair follicles known to precede their appearance might take place. In view of the distinctiveness of the stage I preneoplasia, any positive finding will be decidedly easier to evaluate as a tumor induction than would any other so-called preneoplastic changes described for other tumors, *e.g.*, hyperplasia, changes in stainability, and cell size.

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DISCUSSION

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The investigations Dr. Algard has reported serve to remind cancer researchers they cannot ignore the influences that stromal and parenchymal cells exert on one another, not only during organ development and functional life, but also during certain phases, at least, of neoplastic evolution. Embryologists, led by Borghese (*1*), Grobstein (*2*), Auerbach (*3*), McLoughlin (*4*), and others, have shown that the isolated epithelium of salivary gland, thymus, skin, and other organ rudiments is *not* biologically the same as the epithelium from the same sources kept in its customary relationship with its own more or less specific mesenchyme.

Dr. Algard now has produced evidence that in certain dependent tumors a similar circumstance exists, and that we may be skipping over a big part of reality when we speak of "hormone-dependent" or "hormone-responsive" tumors. Even the term "permissive" is hardly adequate, for the neoplastic cells seem not to be directly dependent on the hormones; they appear to be regulated by the *combination* or "complex" of hormone plus an intact relationship with stroma, inasmuch as the manifestation of hormone dependency is in turn dependent on the maintenance of the relationship with stroma. Perhaps we have tended to overlook this intermediation by the stroma because, under ordinary conditions, the presence of the stroma can be taken for granted.

One is forced to wonder, however, if the stroma in Dr. Algard's trichoepithelioma system functions *only* in a restrictive capacity. It is unlikely that the stroma of the normal flank organ acts only as a suppressor, for if it did one would expect the normal epithelium to grow without restraint when separated from its dermal mesenchyme. Perhaps results of the work Dr. Algard has outlined for the future will help to clarify this point. At any rate, we should be prepared to consider the possibility that stroma may act either as a growth-suppressing or as a growth-promoting factor, depending on prevailing conditions. Certainly in the course of embryonic development a positive influence is exerted by certain mesenchymes on their respective epithelial associates.

In the system Dr. Algard has described for the dependent flank-organ tumor of the hamster, it must be presumed that some enduring alteration has taken place in the epithelium of the stage III tumors. Otherwise these tumors would not be able to respond to androgen plus estrogen after lying dormant in untreated hosts. Is this enduring change in the epithelium equivalent to initiation in Berenblum's two-stage concept (*5*) of carcinogenesis? If so, is the action of androgen plus estrogen then equivalent to that of a promoter? Since the stage I and stage II lesions can be induced by androgen alone, one would have to look on androgen both as an initiator and as part of a promoter complex if it is to be fitted into the two-stage induction concept. If Dr. Algard's concept is correct that the influence of sex hormone is mediated by the stromal cell, then one must wonder whether the hormone itself is either promoter or initiator, or whether some as yet unidentified substance produced by the stromal cell responding to androgen is the real proximal agent, acting both as initiator and promoter. It is quite apparent that many theories can be constructed to fit the information as it now stands. Perhaps the two-stage concept cannot be applied to this system at all. My object here is only to raise the question as to whether stromal cells or some product of them may act as initiators, promoters, or both.

The function of the stroma in the phenomena of tumor dependency and responsiveness has been examined by a number of investigators. Outstanding is the work of DeOme and his associates, who have shown the essentiality of a bed of mammary fat (*6*) or white fat at other sites (*7*) for growth of hyperplastic mammary nodules in mice. Bearing closely on DeOme's observations are those of Lasfargues (*8*), who has reported that the Bittner milk agent is unable to propagate in cultures of isolated mammary-gland epithelium, though it can do so in intact mammary tissue in organ cultures. Other experiments probing stromal-epithelial relationships are those of Van Scott and Reinertson (*9*) working with human basal-cell epitheliomas. They observed that pure isolates of tumor epithelium from basal-cell epitheliomas were unable to survive when transplanted into the subcutis, though basal-cell epithelioma with stroma intact was able to survive and grow when transplanted to the subcutis.

As I have raised questions concerning a possible role of stroma in initiation and promotion phases of tumor induction, I am obliged to emphasize at this point that there may be danger of clouding our thinking if we fail to distinguish between the primary, or at least the very early, events in tumor genesis and the later events concerned with tumor dependency or responsiveness and progression to various stages of autonomy. There may be more overlap of these two areas than we suspect, but it seems fairly clear that Dr. Algard's work, as well as that of DeOme and co-workers and especially that of Van Scott and Reinertson, deals with the influence of the supporting tissue on epithelium that has already gone some considerable distance along one of the pathways to the ill-defined conditions we speak of as neoplasia.

On the other hand, there have been studies, such as those of Orr and his associates (*10*) in Birmingham, which may be interpreted to show that the supporting stroma is essential in the earliest phases of carcinogenesis. I would like here to present some results of experiments designed to test a hypothesis proposed at a symposium on virus tumors 2 years ago (*11*). These experiments were directed toward analysis of one particular tumor-induction system and are not strictly comparable with those of Dr. Algard, which deal with systems of tumor dependency on hormones.

To go back, it was suggested that in certain virus-induced epithelial tumors the stroma might play a critical part in the induction process, and that tumor development might be thought of as dependent not only on viral action, but also on an influence of the stroma on the virus-epithelial cell interaction. This suggestion was based (*a*) on microscopic observations of changes in the mesenchyme as well as in the epithelium of organ cultures of mouse submaxillary gland infected with polyoma virus (*12*); (*b*) on the logic that any factor such as the mesenchyme, which has a specific determining role in the morphogenesis of the salivary gland, should also exert some influence on the response of the salivary epithelium to a virus—particularly to a virus such as the polyoma virus which displays a strong though not strictly specific tropism for the salivary gland in the mouse.

The evidence we now have comes from experiments that were essentially repetitions of Grobstein's early experiments (*2*) with submandibular-gland rudiments, but with the addition of polyoma virus to the system. Submandibular-gland rudiments were separated into their epithelial and mesenchymal components, and the behavior and morphology of these after infection with polyoma virus were compared with the same parameters of polyoma-infected rudiments that were left intact or were re-formed by recombination of the trypsin-separated components. Heterospecific reciprocal recombinations with rat and mouse submandibular rudiments were also used, as I was especially curious to see if rat salivary-gland epithelium might be transformed to neoplastic cells by polyoma virus in the presence of mouse mesenchyme.

Some results of these experiments are shown in table 1. As was to be expected from previous work, the intact rudiments showed nuclear changes and cytopathic effect of virus in both the epithelium and mesenchyme, and in late cultures the proliferative response of epithelium was seen. These proliferations of altered epithelium are thought to be equatable with neoplastic growth *in vivo*.

TABLE 1.—Response of isolated or recombined submandibular components to polyoma virus *in vitro*

		Total No. rudiments or isolated components			Response	
Rudiment or combination of components cultured	Number of cultures	Epithelium	Mesenchyme	Intact	Nuclear changes	Proliferation
Isolated submandibular epithelium, mouse	10	77			0	0
Isolated submandibular mesenchyme, mouse	5		44		+	0
Intact submandibular rudiments, mouse	35			136	Epithelium + Mesenchyme +	+ After 30 days
Recombined submandibular epithelium and mesenchyme, mouse	6	59	59		Epithelium + Mesenchyme +	+ After 30 days
Mouse mesenchyme and rat epithelium	12	118	81		Epithelium 0 Mesenchyme +	0
Rat mesenchyme and mouse epithelium	7	81	118		Epithelium ± Mesenchyme 0	0
Intact submandibular rudiments, rat	6			48	Epithelium 0 Mesenchyme 0	0

When the submandibular epithelium was isolated from mesenchyme, it failed to show morphologic transformation or proliferation in response to virus. The isolated epithelium when not infected with virus was likewise unable to proliferate under our culture conditions.

The isolated submandibular mesenchyme of the mouse showed the same cytopathic effects, including nuclear inclusions, that were seen in intact rudiments infected with polyoma virus. No tumor-like proliferation of the mesenchyme was seen.

When trypsin-isolated epithelial and mesenchymal components were recombined in the presence of virus, the epithelial response was again the same as in intact rudiments. The heterospecific recombination of mouse epithelium with rat mesenchyme did not result in any alteration of the response of rat submandibular epithelium to polyoma virus. It showed neither a cytolytic nor a proliferative response. In the reciprocal of this recombination, the mouse epithelium occasionally showed nuclear changes associated with virus effect, but no proliferation.

These results seem to be clear-cut. If the rudimentary submandibular epithelium is separated from its mesenchyme, it is no longer converted to neoplasia by polyoma virus *in vitro*. But would the same be true *in vivo*?

We are approaching the answer to this question by making use of a new experimental tool that we recently reported (*13*). If one infects a submandibular rudiment with polyoma virus and transfers it immediately to the subcutaneous tissues of an isologous newborn mouse, a salivary-gland tumor of the polyoma type appears at the site of transfer in about 75 percent of the recipients. The use of F_1 hybrid recipients has allowed us to demonstrate by transplantation tests that these tumors originate from the infected donor tissue and not from the secondary host.

This finding made it possible to perform the epithelial isolation experiment *in vivo*. Still in progress, this experiment is being carried out just as in cultures, except that after the epithelium and mesenchyme are separated from one another with trypsin and infected with the virus, each in pure form is transferred subcutaneously to a histocompatible newborn mouse. Intact infected rudiments and recombined infected rudiments serve as controls.

Table 2 shows the results to date. From this it is apparent that, thus far, the experiments *in vivo* confirm those *in vitro*. When the epithelial and mesenchymal components were isolated, infected, and transplanted separately, no tumors appeared. When the

TABLE 2.—Tumor development in polyoma-infected transplants of submandibular rudiments or isolated components

Transplant	Number of rudiments or components of rudiments	Number of newborn recipients	Number developing salivary-gland tumors at transplant site	Percent
Intact submandibular rudiments	58	15	11	73
Submandibular epithelium alone	74	15	0	0
Submandibular mesenchyme alone	79	18	0	0
Submandibular epithelium and embryonic dermis	4 (epithelium)	2	0	0
Embryonic epidermis and submandibular mesenchyme	4 (mesenchyme)	2	0	0
Intact embryonic skin	15	3	0	0

rudiments were infected and transplanted intact, tumors appeared at the site of transfer in 11 of the 15 recipients. We have only recently started the recombination experiments and have no results on them yet.

How are these findings to be interpreted? So many theoretical possibilities can be advanced that it would be extravagant to try to cover them all here. However, some major ones might be considered:

1) To reach the specific intracellular site that it must achieve to induce neoplastic transformation, the virus might require, as a sort of track or guideline, the active interplay of specific epigenetic (salivary-gland stroma) and genetic (mouse genome) factors. This concept might also require that a specific type of nuclear differentiation, brought about and maintained by epigenetic influences, must provide some strategic condition necessary for neoplastic transformation by virus. Thus, cells with other types of nuclear differentiation might support viral reproduction, but would not be susceptible to neoplastic transformation.

2) Contact between stromal and epithelial cell might be necessary for transduction of essential information by virus passing from mesenchyme to epithelium.

3) *After* virus has infected the epithelium, the response of the epithelium might depend on the sort of support or lack of support it receives from its stroma. A competitive situation between epithelial cell and virus might be created in which the cell could proliferate and block viral replication only as long as it received proper support (nutrition?) from its stroma. Lacking this, the cell would be unable to compete with virus and virus would multiply at the ultimate cost of host cell destruction. This concept would fit well with the observations of Noyes and Mellors (*14*) on cell-virus relationships in the Shope rabbit papillomas.

4) Viral infection of the epithelium might be entirely unnecessary for tumor induction. Neoplastic transformation might be caused by production of an "abnormal inducer" by stromal cells infected with virus. In this scheme, viral action would be indirect, requiring mediation by a nonviral oncogenic factor produced by the stroma.

5) As Dr. Algard has suggested for his hormone-dependent systems, the influence of stroma might be restrictive, so that the cytolytic effect of virus on mesenchyme would serve simply to release proliferative activity of the epithelium.

I shall not go into the arguments *pro* and *con* these concepts. Some are apparent to us now and others will certainly be revealed with further experiments. Suffice it to say there is an increasing awareness in many laboratories that the stroma of epithelial organs must be reckoned with in carcinogenesis and tumor growth, whether the process is started by a hydrocarbon, a virus, or a steroid hormone. This may be a circumstance peculiar to epithelial tumors because epithelium is dependent on, or is strongly conditioned by, its stroma. However, we have the leiomyomas observed by Dr. Algard as possible examples of stroma-controlled connective-tissue tumors. Even so, we should continue to suspect and test the possibility that even a single tumor agent may operate through different mechanisms when it induces sarcomas than when it induces epithelial tumors. And more than ever we are cautioned by Dr. Algard's work and that of other participants in this symposium to recognize that epithelium in either short-term or continuous monolayer cultures deserves to be distinguished from epithelium of like origin, as it exists *in vivo.* Until organ-culture methods reach a higher level of perfection, we must grant also that epithelium in organ cultures does not long remain identical with that *in vivo.*

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ORGAN-CULTURE ANALYSIS OF INHERITED RETINAL DEGENERATION IN RODENTS[1, 2]

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SUMMARY

Inherited retinal dystrophy features progressive and selective loss of photoreceptor cells. Pathogenesis differs in mouse and rat. Organ cultures of intact 8- to 10-day-old mouse eyes show differentiation of rod outer segments when retinene is added to the medium. Outer segments were identified by characteristic staining properties and by electron microscopy. Glucose-U-C^{14} and serine-H^3 incorporation was demonstrated autoradiographically in all eye tissues. Incorporation was greatest in developing rod outer segments. Dystrophic eyes did not form rods; the disease was expressed in culture essentially as *in vivo*. A to-and-fro migration of cell nuclei in relation to mitosis of photoreceptor cell precursors is known *in vivo* and was demonstrated with thymidine-H^3 and autoradiography in cultures of 2-day-old mouse eyes. Mitotic events and migrations were similar in cultures of normal and dystrophic mouse eyes. No expression of disease was uncovered before time of outer segment formation. Rat retinal dystrophy develops differently from mouse dystrophy. In the rat an extracellular lamellar material containing rhodopsin forms simultaneously with rod outer segments, and lies between them and the pigment epithelium. Later, photoreceptors degenerate. Neither rods nor extracellular material were synthesized when rat retina and pigment epithelium were cultured separately. Pigment epithelial cells in pink-eyed dystrophic rats developed periodic acid-Schiff-positive cytoplasmic granules and then formed yellow-brown pigment *in vitro* but not *in vivo*. This finding, not seen in albino controls, probably reflects a mutant pigmentation gene, independent of the dystrophy gene. The mutant pigmentation gene impairs pigmentation *in vivo* far more than *in vitro*, whereas the mouse dystrophy mutation is expressed *in vitro* and *in vivo*, and appears to be intrinsic to the cultured cells.—NCI Monogr. No. 11: 227–246, 1963.

INHERITED RETINAL dystrophies are characterized by progressive and selective loss of photoreceptor cells. Late changes include migration of some pigment epithelial cells into the retina and proliferation of retinal capillaries into the pigment epithelial cell layer. The condition is known in mouse, rat, dog, and man [reviewed in (*1*)].

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In the past year it has become evident that the disease develops differently in rat and mouse, even though the end result is very similar in the two species (*1*). The mouse disease sets in during the early part of the 2d postnatal week, when all the retinal cell types have formed and the rod outer segments have just begun to differentiate. At that point, rod outer segments cease to develop and over the ensuing days the whole photoreceptor cell degenerates without recognizable phagocytosis of the products of degeneration. Normal vision is never attained. In the dystrophic rat, on the other hand, the disease is first recognizable late in the 2d postnatal week, when an extracellular lamellar material appears between the pigment epithelial cells and the developing rod outer segments. As observed histochemically and under the electron microscope, this material has the properties of rod outer segment substance, except for a less regular arrangement of its constituent lamellae. Like rods, it contains the visual pigment, rhodopsin. The rod outer segments themselves develop normally at the same time that the extracellular material is forming. By 18 days of age, visual sensitivity has reached virtually adult levels. Since both the lamellar material and the normal rod outer segments contain rhodopsin, the dystrophic rat eye at 18 days may contain twice the normal rhodopsin content. The rhodopsin has a normal absorption spectrum and forms the usual products when bleached. The mouse disease properly is termed an aplasia, the rat disease a dystrophy (*1*); for convenience both are referred to here as dystrophies.

No metabolic basis for the degeneration of the photoreceptor cell is known, and it is not even certain whether retinal dystrophies in the several mammalian species represent one basic disorder with somewhat different expressions, or several disorders with a common end stage. The attractive suggestion has frequently been made that the disease could result from defective metabolism of vitamin A or one of its derivatives. However, the wealth of available data relating vitamin A to retinal structure and function may yet prove misleading, for the disease could as well reflect some other primary metabolic defect not so readily analyzed at present. In view of these uncertainties, it seemed worth while to challenge the dystrophic retina at different stages of its development by means of tissue culture, in the hope that further clues to the nature of the abnormal cell behavior might be forthcoming. This report reviews these exploratory studies, portions of which have been presented elsewhere (*2, 3*).

MATERIALS AND METHODS

Intact eyes of 8- to 10-day-old C3H mice were cultured by Trowell's method (*4*) on Millipore rafts in an atmosphere of 95 percent O_2 and 5 percent CO_2. This inbred strain of mice carries the recessive mutant gene, retinal degeneration, *rd*. Noninbred Swiss albino mice were used as controls. In another series of experiments, eyes of 2-day-old mice of the same stocks were cultured in room air. Since the disease is expressed in all individuals of the affected strain, the eye of a young animal may be described as "potentially dystrophic" even before the dystrophic changes are evident.

The dystrophic rats were pink-eyed, tan-hooded animals derived from the colony maintained at the Royal College of Surgeons, London (*1*). Eyes were removed at 10, 13, 18, and 30 days of age and segments of retina or pigment epithelium-choroid-sclera about 2 mm square were explanted, either in room air or in 95 percent O_2 and 5 percent CO_2 (*4*). Control tissues were obtained from Sprague-Dawley albino rats (Charles River Breeding Co., North Wilmington, Mass.) of the same ages.

The culture medium was a mixture of horse or human placental serum plus synthetic medium 1066 (Connaught Laboratories) or NCTC 109 (Difco) plus Tyrode's solution containing 1 percent glucose. Serum, synthetic mixture, and Tyrode-glucose were combined in a 1 : 2 : 1 ratio. The mixture was supplemented with insulin at a final concentration of 0.1 unit per ml. In many series the media were supplemented with crystalline all-*trans* vitamin A, all-*trans* retinene, or 11-*cis* retinene, each dissolved freshly in ethyl alcohol and added to the culture medium to give a final carotenoid concentration of 20 μg per ml and a final alcohol concentration of 0.2 percent (*2*). In some series, radioactive metabolites (New England Nuclear Co.) were added to the medium at the following final concentrations: thymidine-H^3 (S.A. 5.0 mc/mM), 0.1 μc per ml; uridine-H^3 (S.A. 600 mc/mM), 0.1 μc per ml; sodium acetate-H^3 (S.A. 100 mc/mM), 0.8 μc per ml; D-glucose-U-C^{14} (S.A. 1.4 mc/mM), 0.5 μc per ml; serine-H^3 (S.A. 33 mc/mM), 2 μc per ml.

Cultures were fed every 3 days and maintained for periods up to 8 days. At the end of the culture period, tissues and their supporting Millipore rafts were fixed overnight in 10 percent acrolein in phosphate buffer, *p*H 7.6, at 4° C. Specimens were dehydrated in 3 changes of methanol: methyl Cellosolve (1 : 1) and embedded in polyester wax (*5*). Some specimens were postfixed in osmium tetroxide, dehydrated, and embedded in Epon for electron microscopic study. Sections for light microscopy were cut at 5 μ and stained with the periodic acid-Schiff (PAS) method or the combined alcian blue-PAS method used in previous retinal studies (*6*). Autoradiograms were prepared with Kodak NTB 3 bulk emulsion by minor modifications of the dipping method (*7*); after exposure times of 1 to 4 weeks, slides were developed and stained with toluidine blue. Thin sections for electron microscopy were mounted on carbon-coated grids and stained overnight on a saturated solution of uranyl acetate dissolved in 50 percent ethanol. They were examined in an RCA EMU 3-D electron microscope.[3]

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[3] These electron microscopic studies were carried out jointly with Dr. John Dowling

RESULTS

Eight- to Ten-Day-Old Mouse

At this stage of development *in vivo*, almost all the retinal cells have formed, and the photoreceptor outer segments are differentiating. The pigment epithelial cells are almost all present. An interstitial mucin stainable with alcian blue occupies the small space between pigment epithelial cells and the developing rod outer segments.

Eyes cultured in the presence of retinene were previously shown to contain material staining strongly with the PAS method and tentatively identified as short rod outer segments (*2*). Cultures maintained in media containing vitamin A or no added carotenoids lacked the PAS-positive material. Dystrophic eyes lacked PAS-stained material even when retinene was added to the culture medium (*2*).

These data were incomplete in certain respects. The identification of the PAS-positive material as rod outer segments was made equivocal by the subsequent demonstration that a PAS-positive extracellular lamellar material, different from rod outer segments, is produced in dystrophic rat retinas *in vivo* (*1*). The material in the cultured normal mouse eyes might possibly be similar.

A representative culture, maintained for 5 days *in vitro* in the presence of retinene, is shown in figure 5. The faintly outlined Millipore raft occupies the *right lower corner*. The folded specimen contains all cell components of retina, pigment epithelium, choroid, and sclera. Retinal ganglion cells and some bipolar cells often appear less healthy than the photoreceptor cells and at times these layers contain intensely PAS-positive elements not seen *in vivo*. Another PAS-positive material occupies the site where one expects to find developing rod outer segments. At the *left lower corner* the retina has separated from the pigment epithelium and an accumulation of amorphous interstitial substance, stained with alcian blue, occupies the space between retina and pigment epithelium.

Other cultures photographed at higher magnifications give conclusive evidence that rod outer segments are indeed found in eyes cultured in media containing retinene (fig. 2). Strongly PAS-positive material organized in parallel rods about 5 μ long appears between rod inner segments and the pigment epithelial cells. The outer ends of these rods are enclosed in a sheath of amorphous alcian blue-stained interstitial substance. The apical microvillous processes of the pigment epithelial cells stain purple and are readily distinguishable from the blue interstitial substance and red rods. These features identify the red structures as rod outer segments. The noncultured control eyes and cultures maintained *in vitro* for only 1 hour stain similarly, but the rod outer segments are shorter on the average (fig. 1). Eyes cultured for 24 hours or more without added retinene show no PAS-positive material at this site. As final proof, the PAS-positive, rod-shaped material seen in the light microscope appears as typical rod outer segments in the electron microscope (figs. 6 and 7). The disc membranes are parallel to one another and are enclosed within a surface membrane. A connecting cilium links these outer segments to the photoreceptor inner segments. By contrast, the excess PAS-positive substance of the dystrophic rat eye appears in electron micrographs as swirling sheets of lamellar material adjacent to the apical processes of the pigment cells and is not enclosed within a cell membrane (*1*).

A second problem in the earlier study was that one could not determine securely whether the presence of rod outer segments in cultures of eyes of the normal mouse indicated new synthesis *in vitro* or maintenance of rod material present at the time of explantation. This problem has now been studied by the addition of labeled metabolites to the culture medium and by the preparation of autoradiograms of tissue sections.

Silver grains in the autoradiograms represent labeled substrates incorporated into the tissues in forms not removed by the fixative, dehydrating solvents, wax, and subsequent alcoholic and aqueous solutions. Cultures maintained for 2 hours in the presence of glucose-U-C^{14} show relatively little uptake of labeled substrate. However, after 2 and 5 days *in vitro*, uptake is marked in all parts of the photoreceptor and pigment epithelial cells, especially in the outer segments. In the normal eye, uptake of isotope after 2 days *in vitro* ranges from about 3 times background in the outer nuclear layer to 7 times background in rod outer segments (table 1). The outer segment is not inert *in vitro*; turnover or, more probably, net synthesis of rod substance is taking place. Similar evidence of metabolic activity in rod outer segments and other tissue components was obtained when serine-H^3 was added to the culture medium.

TABLE 1.—Glucose-U-C^{14} incorporation in cultures of eyes of the mouse

Tissue compartment	Grain counts per unit area ± SD*	
	Normal	Dystrophic
Outer nuclear layer	16. 2 ± 4. 1	26. 6 ± 3. 9
Rod inner segments	29. 3 ± 7. 5	46. 2 ± 7. 4
Rod outer segments	37. 3 ± 9. 9	—
Pigment epithelium	27. 4 ± 6. 7	(†)
Choroid	24. 8 ± 4. 7	(†)
Background	5. 3 ± 1. 6	4. 4 ± 1. 4

*Counts were made on 1 normal and 1 dystrophic eye taken from 10-day-old mice and maintained in culture for 2 days in medium containing glucose-U-C^{14}, 0.5 μc per ml. Each number is the average grain count for 10 areas, each 70 × 5 μ, as recorded with the aid of an ocular grid. Autoradiograms were exposed for 6 days.

†Pigment epithelium and choroid in C3H dystrophic mice are labeled, but the high concentration of melanin pigment interferes with accurate counting of silver grains.

In dystrophic eyes, on the other hand, no rod outer segments are seen with the light microscope and only rare ones with the electron microscope, despite the presence of retinene in the culture medium. The rod inner segments lie directly apposed to the pigment epithelium. Cilia and other components of the inner segments look normal, as do the pigment epithelial cells (figs. 8 and 11).

Labeled metabolites are incorporated into choroid, pigment epithelium, and all the photoreceptor cell parts which are present in the dystrophic eye (table 1). The higher counts in the dystrophic specimen compared to the control are of uncertain significance in the absence of many additional samples, since the autoradiographic technique is not yet adequately refined for quantitative purposes. Comparison of different tissue compartments within a given section is more reliable.

Two-Day-Old Mouse

In the 1st postnatal week *in vivo,* most photoreceptor cells and many cells of the bipolar layer are just forming by mitotic division. They are among the last neurons to form, but they develop by essentially the same mechanisms as neurons elsewhere in the embryonic nervous system. Cell nuclei make deoxyribonucleic acid (DNA) while located deep within the retina and then migrate to the outer retinal surface, adjacent to the pigment epithelium, for mitosis. Following mitosis they migrate inward again, to assume their proper position in one of the retinal nuclear layers. Thus on the 2d postnatal day, most of the nuclei in the outer half of the retina are in motion, some migrating toward the outer surface and others away from it (*8*).

The same migrations occur *in vitro* (*3*). Autoradiograms of cultured eyes exposed to thymidine-H^3 for 1 hour *in vitro* show a band of labeled nuclei about 5 to 10 nuclear layers from the outer retinal surface (fig. 9). After 12 hours in culture, most of the labeled nuclei are in the outermost third of the retina and most cells in mitosis have labeled chromosomes (fig. 10). The number and distribution of labeled cells are comparable *in vitro* and *in vivo,* though the rate of migration is about 50 percent slower *in vitro*. No significant differences in DNA synthesis or in the pattern or rate of migrations were detected between normal and potentially dystrophic retinas.

In a further search for expression of genetic differences prior to the arrest of rod outer segment formation, uridine-H^3 was added to the culture medium. Uridine-H^3 incorporation into ribonucleic acid (RNA) is equivalent in culture of normal and dystrophic eyes. Acetate-H^3 uptake also is comparable.

Rat Retina

The pace of the dystrophic process in the rat retina *in vivo* is slower than in the mouse. Rod outer segments have just begun to form in retinas of 10-day-old rats, and have reached 50 percent of the normal adult length at 18 days. The rate of rod formation is the same in dystrophic rats, but simultaneously an extracellular lamellar material, not seen in normal retinas, appears between the rods and the pigment epithelial cells. This extracellular material, like rod outer segments, contains rhodopsin. The quantity of the extracellular material is greatest at the end of the 3d week *in vivo* (*1*).

During dissection of the tissues just before explantation, the retina separates from the outer tissues of the eye (pigment epithelium-choroid-sclera). The two regions were cut into 2 mm squares and cultured separately or together for periods up to 8 days. Even when the retina is repositioned against the pigment epithelium, the two tissues never reestablish the intimate contact they had *in vivo*.

As described by Lucas and Trowell (*9*), the isolated retinas of 10- and 13-day-old rats survive reasonably well *in vitro* but fail to synthesize new rod outer segment substance. In electron micrographs normal cilia are seen emerging from the rod inner segments but, in contrast to the mouse results, no synthesis of outer segments was demonstrated even when retinene and labeled substrates were in the medium. In cultures of 18- and 30-day-old retinas, survival is variable and generally poorer than with the younger retinas. Rod outer segments present at the time of explantation lose their stainability with the PAS method and are already degenerating within the first few days *in vitro*.

Organ cultures of pigment epithelium separate from retina have not been described previously. The pigment epithelium survives in an atmosphere of 95 percent O_2 and 5 percent CO_2 or room air, and is healthiest in our cultures when the apical surface lies against another tissue or against the Millipore raft (fig. 12). When the apical surface is freely exposed to the medium, some cells slough off, while others are flattened and pyknotic.

We sought the source of the extracellular rhodopsin-containing lamellar material which the dystrophic retinas produce *in vivo*. This material lies closely in contact with the apical microvillous processes of the pigment epithelial cells *in vivo*, and might originate in these cells or in some retinal element (*1*). No evidence of production of such extracellular material by pigment epithelial cells or by isolated retina was found in the cultures. In cultures of tissues from 30-day-old rats, abundant PAS-positive extracellular material was associated with the pigment epithelium (fig. 12), but we have no evidence that it was synthesized *in vitro*.

Pigment epithelial cells of the dystrophic rat retina behave differently from the normal controls, but the differences probably are not directly related to the dystrophic process. In unstained sections of cultures of 18-day-old dystrophic eyes we were surprised to find numerous yellow-brown pigment granules in the cytoplasm of pigment epithelial cells. In cultures of 3 days' duration of tissues from 10- and 13-day-old dystrophic animals, the pigment epithelial cells commonly contain abundant cytoplasmic granules less than 1 μ in diameter. These granules stain with the PAS method (fig. 3). After 8 days *in vitro* the cytoplasmic granules of the pigment epithelial cells are an endogenous yellow-brown color with a faint pink overlay imparted by the PAS stain (fig. 4). Many cells of the choroid also contain PAS-positive cytoplasmic granules at early stages of culture (fig. 3) and come to contain dark brown and yellow-brown granules after longer periods *in vitro* (fig. 4). The PAS-positive granules apparently represent the pigment granules prior to pigment formation in them. No pigmentation was seen with the light microscope in tissues of the control rats *in vitro* or in tissues of the dystrophic rats *in vivo*.

The identification of the cytoplasmic granules as pigment granules was confirmed in electron photomicrographs, though the nature of the pigment is uncertain. The prominent dense cytoplasmic granules seen with the electron microscope correspond in position and size to the PAS-positive granules. Granules somewhat like these are seen *in vivo* in 40-day and older rats, but nothing comparable is seen in the first several postnatal weeks (*1*).

We interpret these results to indicate another genetic difference between the dystrophic and control rats, apart from the gene responsible for the dystrophy itself. The controls are albino animals homozygous for the *c* gene and unable to form melanin in any cells. The dystrophic rats have pink eyes and a tan "hood" on an otherwise unpigmented coat. Their particular complement of pigmentation genes is not known, but they might have a gene such as the *p* gene, pink-eyed dilution, which can give variable expression (*10*). Pigmentation is known to be influenced by the tissue environment as well as by the genetic constitution (*11*).

DISCUSSION

Lucas and Trowell (*9*) first obtained good survival of postnatal retina in organ culture, and thus stimulated the present experiments. Our data add two points concerning the formation of normal photoreceptor cells. First, the to-and-fro migrations of cell nuclei, so prominent in the early development of photoreceptor cells *in vivo*, also occur in organ culture. These migrations are not significantly influenced by factors extrinsic to the eye. Second, the rod outer segments, the photosensitive parts of the receptor cells, differentiate and show metabolic activity when eyes are cultured in the presence of retinene (vitamin A aldehyde). No rods form in the absence of added carotenoid or when vitamin A itself is added to the culture medium.

The analysis of dystrophic processes is far more difficult. Lucas (*12*) indicated that the degenerative process continues even when the dystrophic mouse eye is isolated in culture, and we have confirmed his results (*2*). In the present studies, cytological and autoradiographic differences between the behavior of normal and dystrophic mouse retinas at the time of rod formation are marked. On the other hand, no expression of this genetic disease has been uncovered prior to the stage of rod outer segment formation.

The rat disease has proved less amenable to analysis by means of organ culture, both because the disease process unfolds more slowly in the rat, and because the rat tissues must be manipulated more extensively before they are explanted. The source of the extracellular rhodopsin-containing lamellar material and its role in the pathogenesis of the dystrophy remain elusive. The data do not allow a decision as to whether rat rods and extracellular material fail to form *in vitro* because a necessary symbiotic relationship between pigment epithelium and retina is lost when the two tissues are separated at the time of explantation, or whether the culture conditions need to be worked out better. The pigment epithelium survives and, curiously, makes pigment far more extensively in culture than *in vivo*, but, as described, this phenomenon is probably unrelated to the dystrophic process.

Organ culture would seem at the present time to have a few uses in the analysis of inherited disease. One role is in the search for metabolic defects. Radioactive metabolites may be used with far less expense in organ cultures than *in vivo*, and the metabolic analysis may be made clearer by the elimination of organs other than the one affected by the disease. Two additional roles, the separation of extrinsic from intrinsic factors and the test of possible therapeutic agents such as retinene, have been discussed (*2*). The present experiment highlights the importance of analyzing mutant disorders in terms of intrinsic and extrinsic factors. The mutation affecting pigmentation in the strain of dystrophic rats is expressed *in vivo* by suppression of eye pigmentation. Yet when isolated *in vitro*, eye tissues form pigment. However, the mutant gene responsible for the dystrophic process in the mouse is expressed *in vitro* as well as *in vivo* and appears to be intrinsic to the cultured eye tissues.

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PLATES

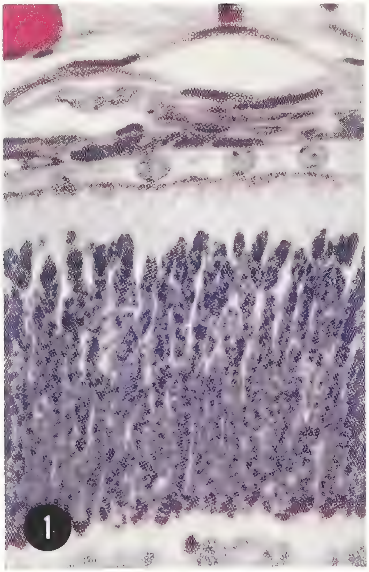
Plate 35

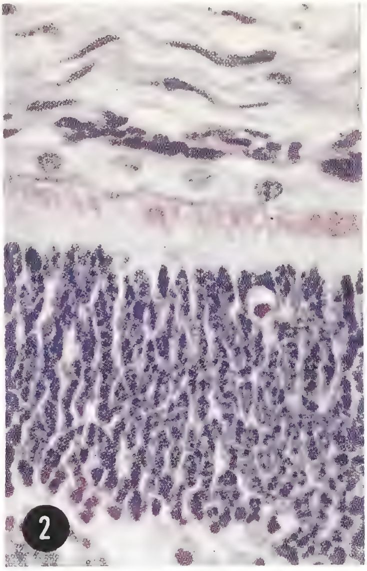
Figure 1.—Normal 10-day mouse eye. Retinal rod outer segments have just begun to form and average 1 to 2 μ in length. They are PAS-positive and readily distinguished from almost unstained rod inner segments *below* and purple-stained apical microvillous surface of pigment epithelial cells *above.* Pigment cell cytoplasm contains few PAS-positive granules resolvable with the light microscope. Alcian blue-PAS. × 750

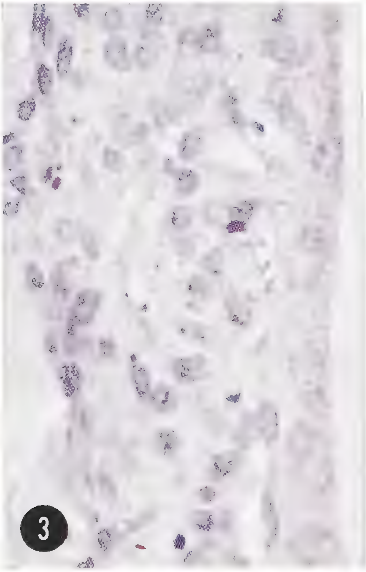
Figure 2.—Other eye of same mouse as in figure 1, maintained in organ culture for 48 hours. Rod outer segments have lengthened to about 5 μ and still stain strongly red with the PAS method. The apical microvillous processes of pigment epithelial cells appear purple because the processes themselves are PAS-positive and are coated with interstitial substance which stains with alcian blue. A few photoreceptor nuclei are degenerating. Alcian blue-PAS. × 750

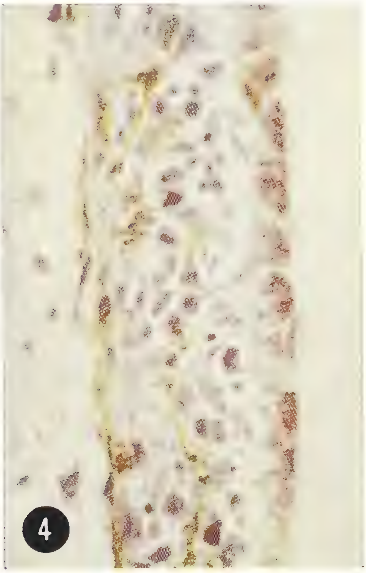
Figure 3.—Pigment epithelium-choroid-sclera of dystrophic 10-day rat maintained for 3 days in organ culture. Cytoplasm of pigment epithelial cells (*right edge* of picture) is filled with PAS-positive granules ranging downward in diameter from about 1 to less than 0.25 μ. *In vivo* the cytoplasm of these cells appears almost empty, comparable to the pigment epithelial cells illustrated for the mouse in figure 1. Scattered cells of the choroid (*left side* of picture) also contain PAS-positive cytoplasmic granules. PAS-hematoxylin. × 750

Figure 4.—Tissues from same eye as in figure 3, maintained for 8 days in organ culture. Cells of pigment epithelium and choroid corresponding to those which contained PAS-positive granules at 3 days *in vitro* (fig. 3), now contain yellow-brown pigment granules. Many cells, especially those of the pigment epithelium, still contain PAS-positive granules as well. PAS-hematoxylin. × 750









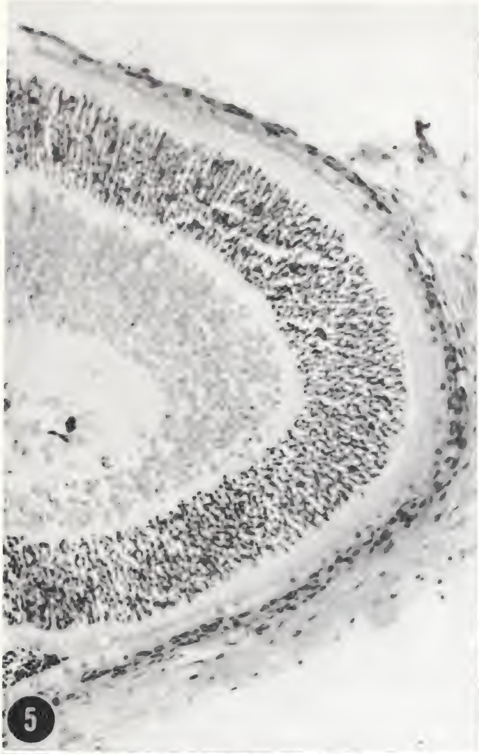
SIDMAN

PLATE 36

FIGURE 5.—Normal 8-day mouse eye, maintained for 5 days in organ culture. *See text* for description. Alcian blue-periodic acid-Schiff. × 190

FIGURE 6.—Normal 10-day mouse eye, maintained for 48 hours in organ culture. Portions of two rod outer segments, about 5 μ long, are shown cut in longitudinal section. The rod discs are well aligned and are enclosed within a surface membrane. Electron micrograph. × 35,000

FIGURE 7.—Another region of specimen illustrated in figure 6. The apical microvillous processes of the pigment epithelial cells (*upper* two fifths of picture) are in direct contact with the obliquely sectioned rod outer segments (*lower* three fifths of picture). The outer segments are oriented in different planes from each other, a finding typical of the developing retina *in vivo* at 10 to 12 days. The surface membrane of the rod is most clearly seen when the outer segment is cut at a right angle to its main axis (*arrow*). Electron micrograph. × 17,500



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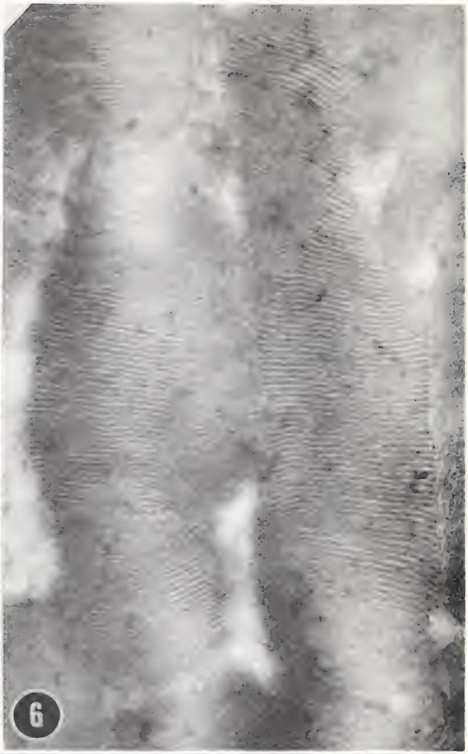

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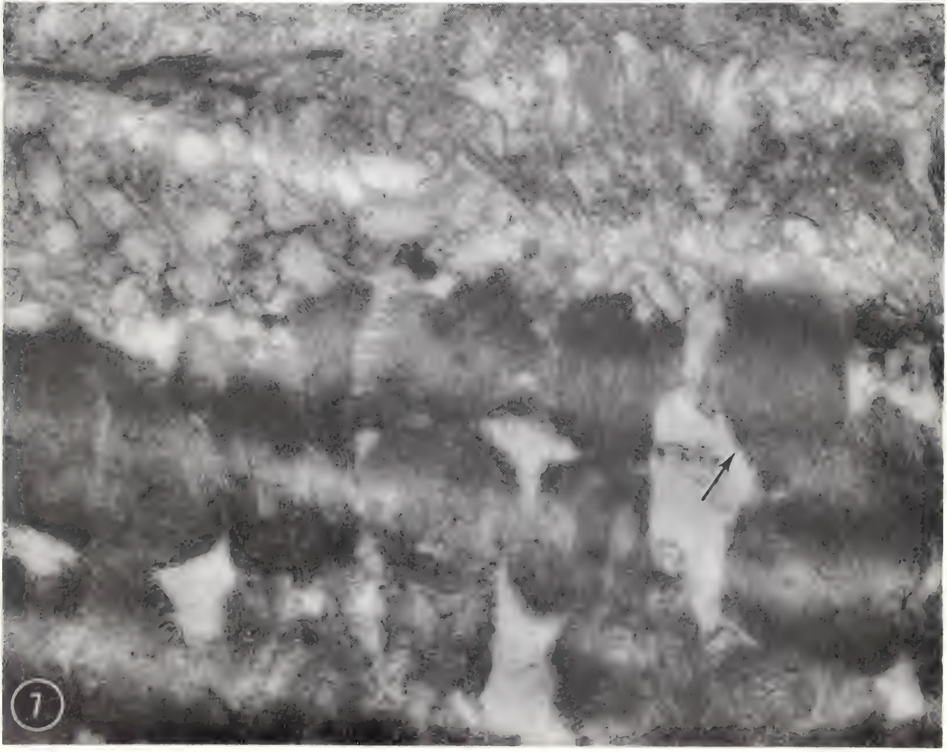

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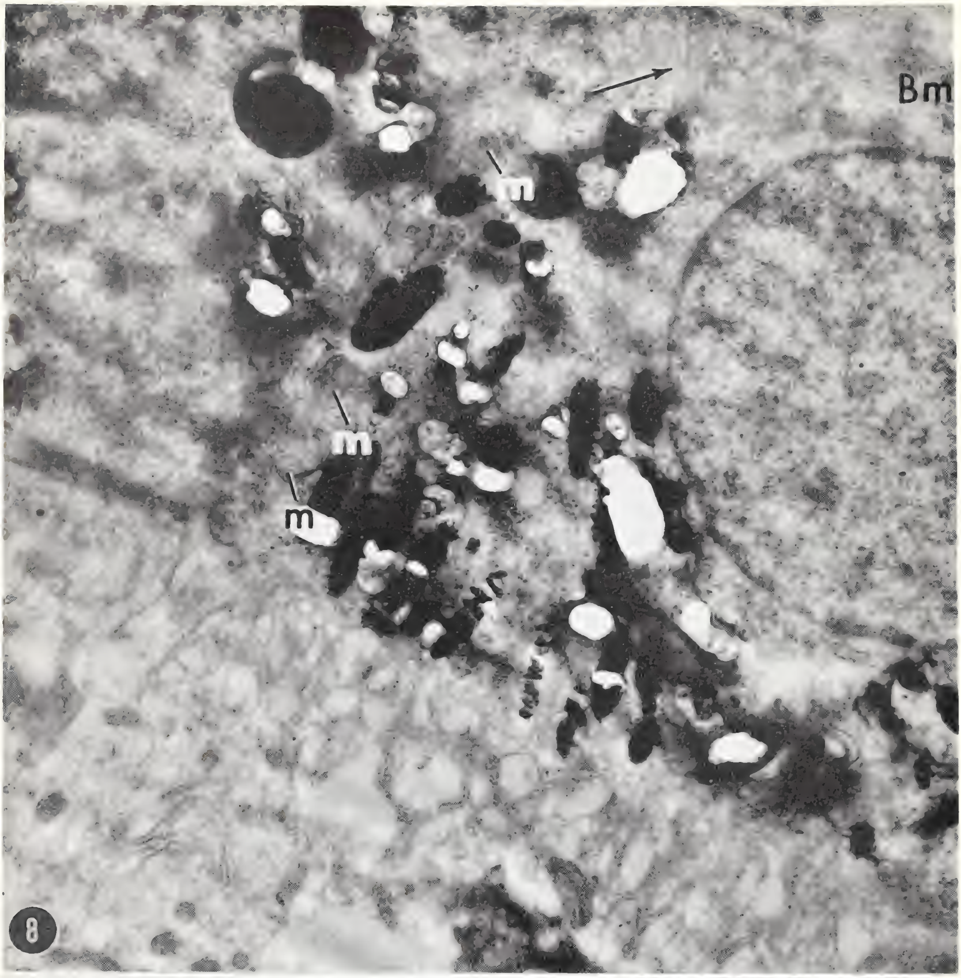
Plate 37

Figure 8.—Dystrophic 10-day mouse eye, maintained for 48 hours in organ culture. All parts of pigment epithelial cell appear normal, including Bruch's membrane (Bm), the highly infolded basal surface (*arrow*), mitochondria (m) concentrated at lateral cell border, and apical microvillous surface. Clear spaces in pigment cell cytoplasm are artifacts produced by displacement of the dense pigment granules during sectioning of specimen. Inner segment of photoreceptor cell (*lower left corner*) abuts directly against apical processes of pigment cells. Traces of lamellar outer segment material are seen to *left* of inner segment and near *mid-lower edge* of picture. Contrast this with abundant normal outer segment material of figure 7. Electron micrograph. × 10,500

Figure 9.—Dystrophic 2-day mouse eye maintained for 1 hour in organ culture with thymidine-H^3 in the culture medium. Labeled nuclei, engaged in DNA synthesis, form a band 5 to 10 nuclear layers from outer retinal surface. Toluidine blue. × 450

Figure 10.—Other eye of same mouse as in figure 9, maintained for 12 hours in organ culture in presence of thymidine-H^3. Labeled nuclei have moved toward outer retinal surface where they divide. Almost all mitotic figures in specimen are labeled. Many ganglion cells, which had formed early in embryonic life, are already pyknotic. Only premitotic and early postmitotic cells of these early postnatal eyes survive *in vitro*, whereas almost all cells of 8- to 10-day-old eyes survive well, *e.g.*, figure 5. Toluidine blue. × 450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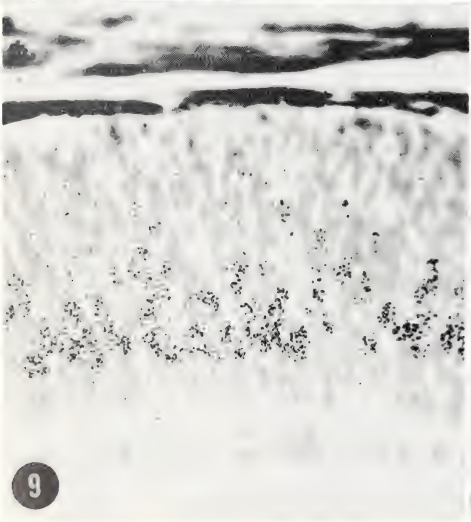
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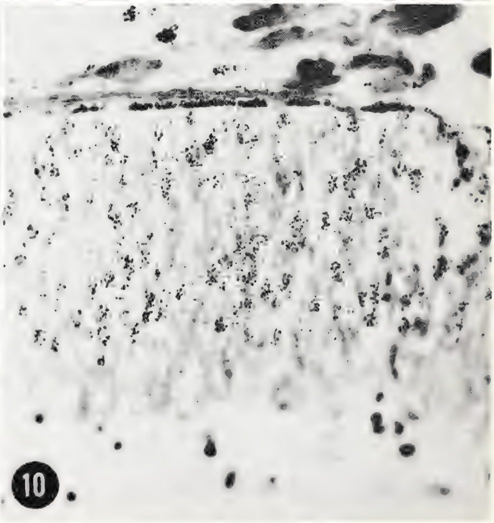
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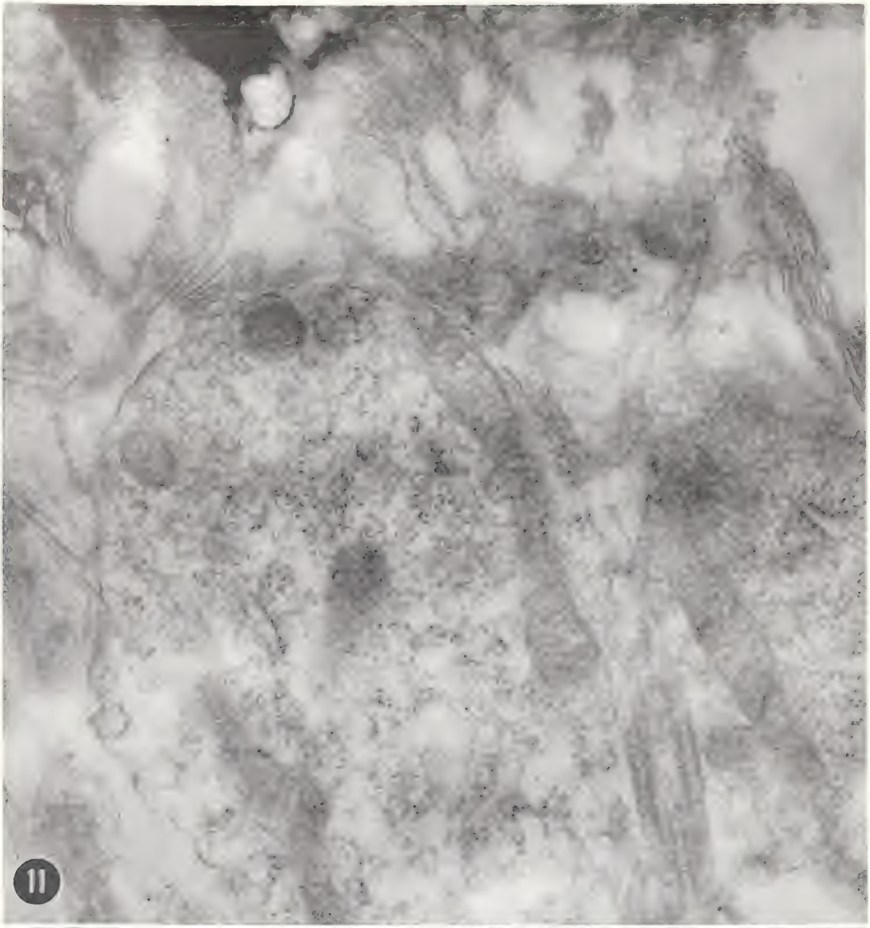
Plate 38

Figure 11.—Dystrophic 10-day mouse eye, maintained for 48 hours in organ culture. A few dense pigment granules in cytoplasm of a pigment epithelial cell are seen in *left upper corner*, and microvillous processes occupy *whole upper quarter* of picture. Two normal-looking photoreceptor inner segments occupy most of rest of picture. They contain the usual concentration of ribosome particles and peripherally distributed mitochondria. A normal cilium in longitudinal section lies between the 2 inner segments. Only abnormality in the section is virtual absence of outer segments. Electron micrograph. × 38,000

Figure 12.—Pigment epithelium-choroid-sclera of dystrophic 30-day-old rat maintained for 5 days in organ culture. Tissue has folded on itself, so that upper and lower halves of picture are almost mirror images. In *right center* is the strongly PAS-positive extracellular debris material enclosed *above*, *below*, and *to left* by a single layer of pigment epithelial cells with relatively clear cytoplasm. This layer in turn is enclosed within choroid and then sclera. Alcian blue-PAS. × 460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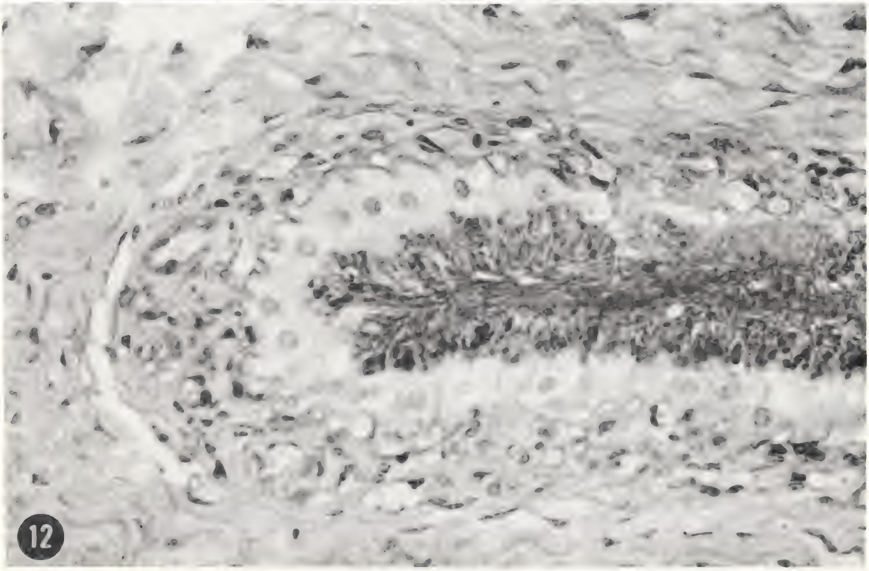
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DISCUSSION

SALOME GLUECKSOHN-WAELSCH, Ph.D., Department of Anatomy, Albert Einstein College of Medicine, New York, New York

I am very happy to have been asked to discuss this interesting paper by Dr. Sidman. As a developmental geneticist, interested in the mechanisms by which genes produce their effects, I am, of course, always partial to any study which attempts to analyze such mechanisms and to trace back the phenotypically noticeable effects of a gene to the first discernible disturbance of a biochemical or biophysical nature. The method of organ tissue culture seems to lend itself particularly well to an analysis of gene effects on development, both normal and abnormal. With the help of this method, one can subject to experimental test those hypotheses derived from mere observational studies of genetically controlled abnormal differentiation. Along these lines, some years ago in a study of the mechanisms of abnormal differentiation, we used organ tissue culture methods for the analysis of an inherited kidney abnormality in mice. I should therefore like to add to the objectives of organ tissue culture studies in the investigation of inherited anomalies listed by Dr. Sidman, the analysis of the role of genes in the interaction of tissues during differentiation.

There are a few specific points in Dr. Sidman's paper on which I would like to comment. Dr. Sidman reported that the retinal dystrophy becomes noticeable during the early part of the second postnatal week, at a time when the rod outer segments have just begun to differentiate. In Dr. Sidman's own words, the photoreceptor cells just fade away, that is, they never complete their differentiation. Dr. Sidman's *in vitro* studies demonstrated autonomous behavior, *i.e.*, failure of the retina from dystrophic eyes to form rods in contrast to normal retinas. In this connection one wonders whether perhaps the actual developmental error responsible for the failure of rod differentiation might not have occurred considerably earlier in development, as Dr. Sidman indicated, perhaps even prenatally so that explantation of the retina at the age of 2 weeks after birth might be too late for a possible reversal of the developmental error even in the presence of retinene. In the mutation we studied which produces kidney agenesis, it could be shown that the kidney rudiments, if explanted early enough in development under appropriate organ-culture conditions, were able to differentiate into kidney elements (*1*); these differentiation potentials are not realized *in vivo*.

Now, what might be the possible nature of the developmental error responsible for retinal dystrophy? In this connection, a few observations reported in the literature might be of interest:

1) Rudnick and Waelsch (*2*) demonstrated a rapid increase in activity of the enzyme glutamotransferase, at the time of maturation of the visual cells in the chick retina of about 17 to 18 days of incubation age. One wonders whether it might not be worthwhile to compare the activity of this particular enzyme in normal and dystrophic retinas from mice. Obviously, the mouse eye shows a pattern of differentiation that is different from the chick, at least in its timing, since it is not nearly as fully developed at birth as the chick eye is at hatching. Further studies of mouse eyes at, or soon after, birth might possibly demonstrate a difference in the activity of this enzyme between normal and dystrophic mice.

2) Moscona (*3*) mentions the changing ability of tissues to reconstruct themselves from dissociated cells at different ages of development. Of particular interest in this connection is the dissociation and reaggregation of retinal tissues. It seems that the ability to reaggregate decreases with increasing embryonic age. However, there is a sudden isolated peak in the descending curve of activity at about 17 days of incubation age. This peak coincides with the time at which the glutamotransferase begins to show a rapid rise of activity, and might indicate a particularly critical stage in retinal differentiation. It appears that disaggregation and reaggregation studies of the dystrophic retinas and a comparison with similar studies of normal retinas might be of some interest.

Another comment which I would like to make refers to the controls used in Dr. Sidman's study. He used noninbred Swiss albino mice as controls. Paigen and Noell (*4*) report that a noninbred population of Swiss mice has approximately 13 percent of animals which are blind as the result of being homozygous for the same retinal dystrophy gene studied by Dr. Sidman. One wonders therefore whether it might not be advisable to use different controls, *e.g.*, normal littermates with the same genotypic background as the mutants studied. These could be obtained by outcrossing the dystrophic mice to normal animals of the same strain with subsequent interbreeding of heterozygotes. Since this of course would make it impossible to differentiate between wild-type homozygotes and dystrophic heterozygotes for controls, one might choose as an alternative control normal C3H mice. However, any of these controls seem to be better suited than the Swiss albino mice which themselves might be blind. There are other reasons why it would be advisable to avoid the use of the albino mutation which might have additional effects on the eye connected with the complete absence of pigment. The values in table 1 of Dr. Sidman's paper, where the colored dystrophic retinas showed a higher uptake of radioactively labeled metabolites than the controls, may be explained by the use of albino mice as controls.

The data obtained by Dr. Sidman with pigment epithelium of the rat are interesting. One should remember that the pigment epithelium, which like the retina originates from the forebrain, forms pigment that is not of melanoblast origin but is produced by polygonal retinal cells *in situ*. Pigment is present in the pigment epithelium in all but the albino mutations. The demonstration of pigment granules, therefore, in the cultures of dystrophic eyes from rats carrying the pink gene is not surprising. Why it appears more rapidly and in larger amounts *in vitro* than *in vivo* remains to be explained. I wonder, however, whether it is necessary to resort to the absence of a suppressor gene in the *in vitro* experiments for such an explanation as Dr. Sidman suggests.

Finally, I would like to comment on a point Dr. Sidman himself has stressed, namely, the difference between the results on mouse dystrophic retinas from those obtained on rat dystrophic retinas. This is yet another demonstration of a principle that cannot be stressed and emphasized sufficiently, that is, that identical phenotypes do not necessarily indicate identical genotypes, whether within a species or between species. There exist a number of gene-controlled hereditary changes in mice which cannot be distinguished from each other in the fully differentiated stage, but which show considerable differences in developmental steps and mechanisms leading to the final abnormalities. Examples for this may be found amply in the literature of developmental genetics of the mouse (*5*).

In conclusion, it seems to me that the search for the developmental error responsible for retinal dystrophy should perhaps be extended into embryonic stages, to a time before the cells of the retina are determined to differentiate into rods. After such a stage it would appear difficult to affect a reversal of the abnormality of differentiation. The analysis of the nature as well as of the timing of the developmental error which differ in mouse and rat retinal dystrophies should yield valuable information, not only about the abnormal but also about normal developmental mechanisms, a goal which I consider to be among the most important in the analysis of any inherited anomaly.

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Dr. Sidman's Response to the Discussion by Dr. Gluecksohn-Waelsch

Thank you very much, Dr. Waelsch, for your instructive comments. You have posed some interesting further experiments.

The retinal degeneration gene, *rd*, is indeed widely distributed in laboratory mouse colonies, but we detected only 1 *rd rd* mouse in 72 of our Swiss albinos, which were selected randomly and examined histologically. The albino animals are very useful for cytological studies on retina and pigment epithelium and were selected originally for this reason. Efforts are under way to establish the *rd* gene on a defined albino background, as well as to provide normal and affected pigmented mice on one genetic background, as you suggest. This takes time, even in mice.

We interpreted our autoradiographic data simply to indicate metabolic activity of normal rod outer segments *in vitro*; the rods are not inert tissue components already present at the time of explantation. The inadequate quantitative reproducibility of the autoradiographic method and the differences in genetic background between control and affected mice both preclude a more refined interpretation of these data at present.

Finally, it is premature to interpret the striking increase in pigmentation of dystrophic rat pigment epithelial and choroidal cells *in vitro* compared to *in vivo*. We do not comprehend suppressor gene action or other mechanisms controlling rates of synthesis in mammalian systems well enough to recognize differences between these mechanisms, much less to choose between them.

STUDIES OF DISEASE IN ORGAN CULTURE: SUMMARY, CORRELATION, AND SPECULATION [1]

JACOB FURTH, Columbia University, New York, New York

This symposium was divided into three sessions: development, function, and disease. My task is to summate the session on disease. A survey of all presentations makes it immediately clear, however, that these three areas are intertwined.

It is true that pathology contributes to knowledge of physiology and vice versa, but investigators have to be cognizant of the limitations resulting from the use of pathologic tissues. Furthermore, in the absence of circulation, homeostatic normalcy does not exist, metabolites are piled up, and some needed substances and cells supplied by distant organs in wanted quantities are not available; so, in a way, all organ cultures are abnormal entities. Often, in lieu of normal cells, tumor cells are used to study function. This is not only permissible but sometimes desirable since neoplastic cells possess some degree of autonomy and also responsiveness to the specific physiologic stimuli of cells of their kind. It should, however, not be forgotten that tumor cells, even the benign types, are abnormal in morphology, function, and responsiveness to physiologic stimuli and that observations made with such cells are not immediately applicable to normal cells. I shall cite a few examples before discussing the four papers of the last session.

Plasmacytoma cells produce proteins and can be used to study protein synthesis. But what proteins? The role of plasma cells is to produce antibodies, yet plasmacytoma hosts are notoriously deficient and not hyperactive in antibody production. The plasma cells seem to be marked cells with limited potentialities.

The behavior of the normal *mast cell* is a veritable mystery but it seems unlikely that study of the mastocytoma cells will solve it, though they are interesting objects of study because they are capable of producing some heparin, histamine, and serotonin *in vitro* and *in vivo*. The function of these tumor cells seems unregulated and the cells do not seem to respond to the physiologic command to release either of these hormones independently. Furthermore, it has been suggested recently that this tumor is caused by and carries a virus.

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[1] Presented at The 13th Annual Meeting of the Tissue Culture Association, Washington, D.C., May 29-31, 1962.

This and viral problems in general bring new vistas to organ cultures but also complications which are difficult to eliminate.

Viral infections present a complex and variable relationship between cells, virus, and their hosts. That of herpes virus is relatively simple, readily reproducible *in vitro*, and different from the other viral infections in which cytopathogenic effects are not evident and the parasitized cells appear normal.

The *thymus* is a fascinating organ for physiologic studies. However, that of the AK mice is abnormal. It carries a virus (probably most other strains of mice do also) that is said to influence differentiation. Furthermore, the AK thymus is said to contain an increased concentration of a lymphocytosis-stimulating factor (Metcalf). In such exciting studies as Auerbach's, the source of thymus should be considered and, for generalization, key experiments are best repeated with rat organs.

The thymus is in the limelight these days and organ cultures seem suitable to unravel some of its mysteries, which were discovered recently in the course of leukemia studies. It should be recalled, in this connection, that the homeostasis of the thymus is poorly known. It is certain that adrenocorticoids bring about lysis of thymic lymphocytes, that the growth hormone stimulates the thymus, and "stressors" cause its involution. The reports that the epithelial reticulum of the thymus is an endocrine organ producing a lymphocytosis-stimulating factor (Gregoir, Metcalf) requires better documentation. Does this epithelial reticulum in adults maintain its lymphocytosis-stimulating capacity after the thymus has undergone involution, as Metcalf believes? I am puzzled by the relation of this epithelium with the reticulum as concerns both histogenesis and origin of lymphocytes and of lymphocytosis-stimulating factors, and by the kind of differentiation these structures undergo during neonatal life. Auerbach's model shows how such problems can be explored with the use of organ cultures.

One advantage of using *functional tumor* cells in place of the corresponding normal cells is that hormone production by them is not blocked as readily by a feedback mechanism as that of normal cells, therefore, they can cause a sustained stimulation of their target cells. A good example of this is the use of parathyroid tumors by Gaillard for reproduction of a bone lesion. Yet one wonders how normal parathyroid would behave. The latter may be inhibited by the products of its activities.

Two of the four interesting contributions of today concern nonneoplastic and two concern neoplastic diseases. All have interesting possibilities.

The demonstration by *Bornstein* of *in vitro demyelination* by autoantibodies is among the major contributions of this meeting. I note with surprise how many presumably normal human sera exhibit demyelinating activity of cultured cerebellum. The correlation of blood values with disease is impressive. Further, it is interesting to note that therapeutic γ-irradiation can cause auto-immune demyelination, that demyelination, as cytotoxicity, requires complement, and that removal of the antibody is followed by remyelination. These findings introduce many important questions, most of which are amenable to precise experimental analysis. Remyelination by removal of the immune globulins may be exploited therapeutically, if successfully demonstrated *in vivo* in experimental animals. Will "globulinopheresis" control the disease or is removal of the antibody-producing lymphocytes and plasma cells necessary, *i.e.* are such disorders lymphocyte-conditioned or dependent?

The nature of the antibody in question, as well as that of the antigen, deserves further studies. Is Bornstein dealing with a new type of antibody? It should be recalled that a single antigen can produce several antibodies, *e.g.*, some highly agglutinating antibodies produced by the same microorganism and directed against flagellae are not involved in cytotoxicity.

Demyelination in multiple sclerosis is probably intermittent and can possibly be gauged by the rise of antibody titer. This calls for a clinical correlation, *i.e.*, search for the possible causes of aggravation of the process. Why are tumors of the central nervous system not associated with demyelination? Is this due to immunologic deficiency of the patients?

Sidman's work is a model analysis of an inherited dystrophy, that of an error of *protein synthesis in the retina.* It elucidates histogenetic events in the normal retina and pinpoints the apparently intrinsic genetic derangement resulting in progressive loss of photoreceptor cells.

Organ cultures can conclusively demonstrate the immunologic basis of a disease or of a deficiency state by its correction as well as causation by virus; but a negative experiment, *e.g.*, failure to implicate the virus is always inconclusive. Witness the errors of outstanding geneticists that indicate the genetic causation of leukemia and mammary tumors in high leukemia and tumor strains, respectively. Nothing in the way of histology or natural history of leukemia suggested a virus until the virus was discovered and linked to some thymic factor, yet to be fully explained. I am awed by the reports that a near majority of animals carry viruses such as polyoma, pleuropneumonia-like organisms (PPLO), and "LDH-virus." Recall the diseases caused in human embryos by the rubella virus.

The two papers related to cancer have tackled some fundamental problems of the neoplastic change and behavior of neoplastic cells. In similar studies cell cultures were misleading though in a way highly revealing. They led to the discovery that *in vitro* cells, which in their multicellular hosts are limited in space and numbers, grow progressively with no apparent restraint. This led to the concept of *contact inhibition* (Abercrombie) which has interested *Leighton* for several years. In the body, environmental forces limit both reproductive capacity and expression of functional capabilities. Organ cultures, *i.e.*, culture of different associated cells, are suitable for analysis of problems of invasiveness and organ affinities. Why do certain cells proliferate in certain organs and not in others? Leighton, extending the work of Wolff, showed the apparently unusual stimulating effect of the mesonephros. More information is needed to explain this. Are there mesonephric-stimulating substances ("trephones") or is this effect related to the structure of the mesonephros, or did a larger number of tumor cells or blood reach the mesonephros under the experimental conditions? Grafting a combination of different organs with the same tumor cells on the chorio-allantois, as discussed by Leighton, may clarify the age-old problem—Why do spleen, kidney, and muscle resist formation of metastases?

The forces underlying contact inhibition remain to be clarified. Earlier the existence of hormone-like substances affecting adjacent cells was postulated, but tumor induction by plastic membranes and prevention of it by perforation of such membranes point to some physical forces.

A major deficiency of organ cultures, the lack of some circulation, will ultimately have to be corrected, but even without it certain factors can well be analyzed in what are now conventional organ cultures, *e.g.*, the embryo is immunologically defenseless. This deficiency is advantageous in studies of the effects of introduced antibodies as well as of normal and "conditioned" lymphoid cells.

In the experiments reported, in which the mesonephros was utilized, mostly old animal tumors were used. Most old transplanted tumors are composed of a mixture of altered clones and some carry viruses, probably picked up in the course of successive passages. It seems desirable to use this technique for the study of natural tumors, including those of man, and also to explore its potentialities for chemotherapy by beginning with cultures of normal and tumor cells placed side by side.

The superiority of organ cultures over cell cultures is well indicated by the work of *Algard.* It seems that in cell cultures, most hormone-producing cells are too busy with reproduction and soon abandon *hormone production* (notable exceptions are Fisher's mast cell tumor, and Sato's AtH tumor culture). Further, cell cultures respond little, if at all, to the physiologic regulators, though the hormone-producing apparatus is retained. In contrast, organ cultures respond to hormones as Algard, Lasfargues, and Murray, and others have demonstrated.

There is a great need for expansion of this type of work for both theoretical and practical purposes. *Hormonal control* of neoplasia in man is in an unsatisfactory state in part because it fails to consider the individuality of the tumor cell, *e.g.*, mammary tumors are known to be variously responsive to hormones, some are inhibited, others are stimulated by estrogens or androgens. For pilot studies we suggest the use of variously hormone-responsive "stabilized" mammary thyroid, thyrotropic and like tumors as developed in our laboratories. The specific hormonal responsiveness of these tumors in organ cultures can be checked *in vivo.*

Although the three hormone-responsive tumors of *Algard* are special to hamsters, they are suitable to study basic principles. Even findings with such an odd tumor as the trichoepithelioma of the hamster may contribute to knowledge of dependency and autonomy and to that of relation of hair growth to sex hormones. Kidney tumors and trichoepitheliomas in other species are not known to be related to hormones.

These hamster tumors are also unique in their androgen responsiveness. Carcinoma of the prostate is the most important human tumor of this kind. Thus far no one (including my associates) has succeeded in establishing a transplantable hormone-responsive adenocarcinoma of the prostate in experimental animals. Frank came nearest to it. The work of Algard suggests that perhaps a combination of estrogens and androgens or the cyclic application of these two hormones is essential for their induction.

In several communications the use of pituitary hormones or the entire pituitary was reported for stimulation of organ culture. There are complications with both. The normal pituitary is a mosaic of several different cell types whose interrelationship and function *in vitro* are poorly known. Each type is subject to specific feedback control. For example, if a normal adrenotrope were used to stimulate the adrenal, it would be inhibited by the corticoids produced. Similarly, the thyrotrope would be inhibited by T3 or T4. Therefore, in such studies as Schaberg presented, functional transplantable adrenotropic tumors would be more advantageous. Similarly, for mammary-gland stimulation functional mammotropic tumors may be useful stimulants. It should be recalled that even extracellular autografts of the pituitary are not physiologic; in these, all but prolactin secretion ceases, while the latter goes on uncontrolled. Recognition of such complications should not be a deterrent, but a challenge to analysis.

Adding hormones in one dose may imitate natural events if the target organ doesn't "feed-back" as in the classical work of Honor Fell on hormonal stimulation of the vaginal epithelium. When Schaberg hyperstimulated the adrenal cortex it underwent necrosis. This is reminiscent of human adrenocortical necrosis which has been attributed to release of AtH by stress. A further study of this situation may be rewarding.

We all know that serum is complex, but for organ cultures in the areas discussed it is more complex than generally believed. It contains pituitary hormones of various sorts in variable quantities. For precise experimentation on growth-promoting effects, I suggest the use of sera from hypophysectomized animals, supplemented with known quantities of wanted hormones. To imitate the physiologic state whereby the product of stimulation inhibits the stimulator, some sort of a perfusion system will have to be worked out.

* * *

In listening to the communications in the past three sessions, I was much impressed by the many unique new possibilities in the study of disease by organ cultures. True, they are still beset with many problems but most of these seem technical and solvable.

In the area of cancer, we look forward to learning about cell homeostasis, contact inhibition in the community of cells, and development of model systems to analyze responsiveness of a given tumor to various hormones so we can predict behavior *in vivo* and guide therapy.

In the area of carcinogenesis we may learn about initiation of cancer, notably by chemicals; this apparently cannot be done in cell cultures.

Neoplastic transformation of cells by virus *in vitro* has already been accomplished with polyoma and chicken sarcoma viruses. With those of lymphoid and other cells, accessory influences may exert a strong influence, *e.g.*, the thymus in lymphomogenesis. The competence of the cells to accept virus is said to be connected with differentiation of lymphoid cells; this too can be analyzed in organ cultures. In general, one should not forget that many cells, normal in appearance, may carry viruses.

This conference, as I see it, will be remembered not as one with new definitive discoveries but as one pointing to many exciting new research possibilities in physiology and pathology by the use of organ cultures. *Doctor Fell*, the originator of organ cultures, modestly called it an "ancillary tool." Yes, it is in some instances, but in others it is presently the major analytic tool at our disposal.

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